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RNA-mediated toxicity in neurodegenerative disease

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Abstract

Cellular viability depends upon the well-orchestrated functions carried out by numerous protein-coding and non-coding RNAs, as well as RNA-binding proteins. During the last decade, it has become increasingly evident that abnormalities in RNA processing represent a common feature among many neurodegenerative diseases. In “RNAopathies”, which include diseases caused by non-coding repeat expansions, RNAs exert toxicity via diverse mechanisms: RNA foci formation, bidirectional transcription, and the production of toxic RNAs and proteins by repeat associated non-ATG translation. The mechanisms of toxicity in “RNA-binding proteinopathies”, diseases in which RNA-binding proteins like TDP-43 and FUS play a prominent role, have yet to be fully elucidated. Nonetheless, both loss of function of the RNA binding protein, and a toxic gain of function resulting from its aggregation, are thought to be involved in disease pathogenesis. As part of the special issue on *RNA and Splicing Regulation in Neurodegeneration*, this review intends to explore the diverse RNA-related mechanisms contributing to neurodegeneration, with a special emphasis on findings emerging from animal models.

Keywords

RNA processing; Bidirectional transcription; RAN translation; RNA foci; Neurodegenerative diseases; Mouse models

Introduction

Neurodegenerative diseases represent a large and heterogeneous spectrum of illnesses caused by the progressive loss of neurons in the central or peripheral nervous system. They can largely be classified into two clinical groups: motor/movement disorders and dementia/cognitive impairments. The motor/movement group can be further classified into clinical subgroups, which include motor neuron diseases, Parkinsonism, ataxia and hyperkinesia. The dementia/cognitive impairments group can be divided into two categories, with cognition/memory deficits characterizing one category and personality, behavioral and language impairments characterizing the other.

While distinct mechanisms contribute to each disorder, it is becoming increasingly apparent that abnormalities in RNA processing represent a common feature among many neurodegenerative diseases. Normal cellular function depends on numerous protein-coding and non-coding RNAs, as well as RNA-binding proteins that associate with RNAs to form ribonucleoprotein (RNP) complexes. Mutations or abnormalities that disrupt RNA or protein components of RNP complexes can be deleterious to cells and cause disease. For instance, some neurodegenerative diseases result from mutated coding and non-coding RNAs, and

misregulation of long non-coding RNA transcription. Multiple mechanisms are now recognized as driving pathogenesis in these “RNAopathies”, including a toxic gain of function caused by RNAs with nucleotide repeat expansions and the formation of nuclear RNA foci, as well as loss of function caused by gene silencing and haploinsufficiency. However, much less is known regarding the mechanisms underlying “RNA-binding proteinopathies”, such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), in which RNA-binding proteins play a prominent role. Such proteins include transactive response DNA-binding protein 43 (TDP-43) and fused in sarcoma (FUS), which form cytoplasmic and nuclear inclusions in disease. It is believed that the abnormal aggregation of TDP-43 and FUS results in a toxic gain of function conferred by the inclusions themselves, as well as a loss of function caused by the sequestration of these RNA-binding proteins. An extensive list of diseases caused by pathological RNA processes is provided in Table 1.

This review, while not intended to provide an exhaustive characterization of all RNAopathies and RNA-binding proteinopathies, aims to highlight the diverse RNA-related mechanisms contributing to disease pathogenesis, with a special emphasis on findings emerging from animal models. Furthermore, we will discuss how our current knowledge of RNAopathies is likely to guide research on *C9ORF72*-linked ALS and FTD (c9FTD/ALS); this is an area of great interest since the recent discovery that a hexanucleotide repeat expansion in the *C9ORF72* gene is the major genetic cause of ALS and FTD (Dejesus-Hernandez et al., 2011; Renton et al., 2011).

Impaired RNA mechanisms in neurodegeneration

Our current understanding of many neurodegenerative diseases has been greatly directed by genetic discoveries. Despite early beliefs that disease-causing mutations are located only in coding regions of genes, it is now well established that mutations in regulatory sequences also influence gene expression and are a significant cause of disease. Indeed, the majority of the transcriptome is composed of noncoding RNAs that participate in numerous physiological activities, such as regulating RNA processing, transcription and translation.

Expansions of noncoding, repetitive DNA microsatellite sequences have been associated with many disorders, and have been of special interest to the neurodegeneration field over the last decade. Among the first nucleotide repeat disorders described are fragile X syndrome (FXS) and Friedreich ataxia (FRDA) (Table 1), both of which are caused by noncoding triplet repeat expansions (Campuzano et al., 1996; Verkerk et al., 1991). These expansions prevent normal protein expression, thus resulting in loss of expression of the fragile X mental retardation protein (FMRP) in the case of FXS, and loss of frataxin expression in FRDA. However, the mechanisms of toxicity are not as straightforward for all neurodegenerative diseases caused by noncoding repeat expansions. Rather, various pathological mechanisms involving RNA, including abnormal translation, generation of antisense transcripts, and the formation of RNA foci that sequester protein complexes and alter mRNA splicing, are involved in disease pathogenesis (Todd and Paulson, 2010).

RNA toxicity

A role for RNA-induced toxicity in microsatellite expansion disorders was first proposed for myotonic dystrophy type 1 (DM1). DM1, an autosomal dominant disorder, is the most common form of muscular dystrophy in adults. While it has variable clinical presentations, features of DM1 include skeletal muscle weakness and myotonia, cognitive dysfunctions, cardiac problems, and cataracts (Renoux and Todd, 2012). DM1 is caused by an expansion of trinucleotide CTG repeats in the 3' UTR of the *myotonic dystrophy protein kinase* (*DMPK*) gene (Table 1) (Mahadevan et al., 1992). The normal size of the CTG repeat varies

between 5 and 30 repetitions, whereas DM1 patients have 50 to 5000 CTG copies (Echeverria and Cooper, 2012).

Soon after the discovery that expanded CTG repeats in *DMPK* cause DM1, investigators set-out to determine whether altered *DMPK* expression contributes to disease pathogenesis. While initial quantitative studies of *DMPK* mRNA and protein in DM1 patient tissues yielded contradictory results, it has since been shown that levels of both sense and antisense transcripts of *DMPK* negatively correlate with repeat length (Fu et al., 1993; Klesert et al., 1997; Thornton et al., 1997). Furthermore, later studies consistently demonstrated decreased *DMPK* protein expression in adult onset myotonic dystrophy and the congenital form of disease (Fu et al., 1993; Hofmann-Radvanyi et al., 1993; Koga et al., 1994; Krahe et al., 1995; Wang et al., 1995). To investigate the consequences of altered *DMPK* protein expression *in vivo*, *DMPK* knockout mice were generated (Berul et al., 1999; Jansen et al., 1996; Reddy et al., 1996). The *DMPK* knockout mice produced by Reddy and colleagues exhibited cardiac conduction defects, late-onset skeletal myopathy, and variations in muscle fiber size, whereas Jansen and colleagues observed only minor and inconsistent changes in head and neck muscle fiber size in their aged *DMPK* knockout mice. Thus, *DMPK* knockout mice recapitulate some, but not all, features of DM1, suggesting the disease is not simply due to loss of *DMPK* protein expression.

In addition to decreased *DMPK* mRNA expression, the repeat expansion in the *DMPK* gene silences the expression of *SIX5*, a flanking gene that encodes a transcription factor (Klesert et al., 1997; Thornton et al., 1997). *SIX5* knockout mice have an increased frequency of cataracts, suggesting that loss of *SIX5* function drives cataracts formation in DM1 (Klesert et al., 2000; Sarkar et al., 2000). Nevertheless, while decreased *DMPK* and *SIX5* expression may account for particular aspects of DM1, other mechanisms of disease must also exist.

In 1995, nuclear *DMPK* RNA transcripts containing CTG repeat expansions were detected, in the form of foci, in fibroblasts and muscle biopsies from DM1 patients, but not from normal individuals (Taneja et al., 1995). Spurred by this finding, the quest began to confirm and decipher the pathogenic nature of mutant RNA. Validating the “RNA toxicity” hypothesis were findings showing that transgenic mice expressing an expanded (CUG)₂₅₀ repeat in the 3' UTR of an unrelated transgene (i.e. skeletal actin) develop myotonia and myopathy, whereas mice expressing a non-expanded CUG repeat did not. These results indicate that transcripts with expanded CUG repeats are sufficient to generate a DM1 phenotype, and support a role for RNA gain of function in disease pathogenesis (Mankodi et al., 2000). It was later shown that RNA foci are formed after the CTG expansion is transcribed into CUGs which induces the formation of a stable hairpin structure enclosing an A-type helix with U–U mismatches and G–C Watson–Crick base pairs. The U–U pairs have a strong electronegative patch and an unusual number of hydrogen bond acceptors, increasing the likelihood of interactions with other RNAs and RNA-binding proteins (Mooers et al., 2005). Indeed, studies aimed at elucidating the mechanisms of RNA toxicity led to the notion that CUG RNA repeats lead to the sequestration and/or altered function of RNA-binding proteins. Two families of alternative splicing factors have been implicated in DM1 based on their ability to bind CUG RNA: the CELF proteins, which include CUG RNA-binding protein 1 (CUGBP1) (Timchenko et al., 1996; Wang et al., 2007), and the muscle blind-like (MBNL) proteins (Fardaei et al., 2002; Jiang et al., 2004; Miller et al., 2000). CUGBP1, a heterogeneous nuclear ribonucleoprotein (hnRNP), regulates splicing of CUG-repeat-containing transcripts (Philips et al., 1998), and translation of C(U/C)G-containing mRNAs (Timchenko et al., 1999). Of particular interest, RNA CUG repeats directly affect expression and activity of CUGBP1 (Timchenko et al., 2001). The formation of CUGBP1-CUG RNA complexes is accompanied by an increased stability of the CUGBP1 protein and, consequently, elevated CUGBP1 expression. Notably, increased

expression of CUGBP1 is observed in DM1 myoblasts, skeletal muscle, and heart tissue (Dansithong et al., 2005; Savkur et al., 2001; Timchenko et al., 2001). In transgenic mice, the overexpression of CUGBP1 in skeletal muscle and heart leads to increased expression of its translational targets, p21 and myocyte-specific enhancer factor 2A, similar to the induction of these proteins in skeletal muscle of DM1 patients (Timchenko et al., 2004). These alterations are associated with muscular dystrophy, an increase of slow fibers, and delayed muscle development. In a second CUGBP1 transgenic mouse model, disrupted splicing of three CELF target pre-mRNAs, *cardiac troponin T* (*Tnnt2*), *myotubularin-related 1 gene* (*Mtmt1*), and *muscle-specific chloride channel* (*Clcn1*) are observed, suggesting that elevated CUGBP1 activity contributes to misregulated alternative splicing in DM1 (Ho et al., 2005).

In contrast to activation of CUGBP1 in DM1, binding of MBNL1 proteins to expanded RNA CUG repeats, which positively correlates with repeat length, causes MBNL1 sequestration and impairs its ability to regulate splicing (Miller et al., 2000). This leads to aberrant splicing of *amyloid precursor protein* (*APP*), *microtubule-associated protein tau* (*MAPT*), and *glutamate receptor ionotropic n-methyl-d-aspartate subunit 1* (*GRIN1*) in the brain of DM1 patients, and altered splicing of *sorbin and sh3-domains containing 1* (*SORBS1*), *doublecortin-like kinase 1* (*DCLK1*), and *calcium/calmodulin-dependent protein kinase II-delta* (*CAMK2D*) in both DM1 patients and *Mbnl1* knockout mice (Suenaga et al., 2012). That *Mbnl1* knockout mice also develop muscle and eye abnormalities characteristic of DM1 provides further evidence that loss of *MBNL1* function plays a role in DM1 (Kanadia et al., 2003).

Along with DM1, RNA foci are observed in various neurodegenerative diseases characterized by non-coding repeat expansions, including the spinocerebellar ataxias SCA8, SCA10 and SCA31, as well as myotonic dystrophy type 2 (DM2), Huntington disease-like 2 (HDL2), fragile X-associated tremor/ataxia syndrome (FXTAS), and the most common pathological subtype of FTD, namely frontotemporal lobar degeneration with TDP-43-positive inclusions (FTLD-TDP) (Table 1). As in DM1, MBNL1 co-localizes with RNA foci in DM2, SCA8, HDL2 and FXTAS (Daughters et al., 2009; Mankodi et al., 2001; Rudnicki et al., 2007; Tassone et al., 2004). In FXTAS, ubiquitin-positive intranuclear inclusions are found throughout the cerebrum and brainstem, and *FMR1* mRNA is found within these inclusions (Tassone et al., 2004). The aggregates also include hnRNP A2/B1, hnRNP G, src-associated protein in mitosis 68 (SAM68), and purine-rich element-binding protein A (Pur), all recognized as RNA-binding proteins (Renoux and Todd, 2012; Sellier et al., 2010). With regards to SCA10, which is caused by expansion of ATTCT repeats in the *ATXN10* gene, hnRNP K is strongly bound by RNA transcripts containing expanded AUUCU repeats. In brains from SCA10 transgenic mice expressing ~500 ATTCT repeats, hnRNP K co-localizes with RNA foci (White et al., 2010). hnRNP K normally plays an important role in splicing, transcription, signaling and apoptosis by binding to RNA through K-homology domains (Bomsztyk et al., 2004). However, these functions are impaired upon the sequestration of hnRNP K by RNA foci. In SCA31, nuclear RNA foci may sequester serine/arginine-rich splicing factors 1 and 9 (Sato et al., 2009). Finally, nuclear RNA foci are observed in frontal cortex and spinal cord tissue of c9FTD/ALS cases (DeJesus-Hernandez et al., 2011), but whether RNA-binding proteins are sequestered by these RNA foci has yet to be reported.

Overall, the “RNA foci-centric” model of disease pathogenesis of DM1 and other repeat diseases is strong. However, there exists reservations that this pathway acts alone (Mahadevan, 2012). Additional mechanisms of toxicity in RNAopathies are discussed below.

Bidirectional transcription

Studies conducted over the past 20 years have revealed the occurrence of extensive bidirectional transcription of eukaryotic genes (Chen et al., 2004; Yelin et al., 2003). Whereas sense transcripts produce functional proteins, antisense transcripts are often thought to play an important role in gene regulation (He et al., 2008; Morris et al., 2008; Yu et al., 2008).

SCA8, an autosomal dominant disorder characterized by cerebellar ataxia resulting from progressive cerebellar neurodegeneration, is caused by a CTG expansion in the 3' end of a non-protein coding RNA, *Ataxin 8 Opposite Strand (Ataxin 8OS)* (Day et al., 2000; Koob et al., 1999). It was thus initially postulated that expression of RNA transcripts with this expanded repeat cause neurotoxicity via an RNA gain of function in a manner similar to that observed in DM1. To gain a better understanding of the pathogenicity of the *Ataxin 8OS* expansion, Ranum and colleagues generated transgenic mouse models expressing a bacterial artificial chromosome (BAC) encompassing the entire human SCA8 locus with either a normal or expanded CTG allele (Moseley et al., 2006). SCA8 BAC-EXP mice with the (CTG)₁₁₆ expansion, but not (CTG)₁₁ control lines, developed a progressive neurological pheno-type. Unexpectedly, they found that two genes spanning the repeat were expressed in opposite directions: *Ataxin 8OS*, which, when transcribed, results in a noncoding CUG expansion RNA and foci formation, and *ataxin 8*, which encodes a nearly pure polyglutamine expansion protein (Fig. 1A). Intranuclear inclusions immunopositive for IC2, a monoclonal antibody specific to polyglutamine expansion tracts (Trottier et al., 1995), were observed in cerebellar Purkinje and brainstem neurons in SCA8 BAC-EXP mice, as well as human SCA8 autopsy tissue. These findings indicate that expression of the ataxin 8 protein forms neuronal inclusions typical of polyglutamine diseases. In a subsequent study, it was found that Mbnl1 co-localizes with the RNA foci of expanded CUG transcripts in cerebellum of transgenic SCA8 BAC-EXP mice (Daughters et al., 2009). Additionally, loss of Mbnl1 in SCA8 BAC-EXP mice enhanced motor deficits and triggered splicing changes, leading to increased expression of GABA-A transporter 4 (GAT4), and loss of GABAergic inhibition within the granular cell layer (Daughters et al., 2009). Based on these findings, both toxic RNA and protein gain-of-function are likely involved in SCA8 (Daughters et al., 2009; Ikeda et al., 2008; Moseley et al., 2006).

Other diseases in which bidirectional transcription may be implicated include FXTAS, DM1, SCA7, and HDL2 (Cho et al., 2005; Ladd et al., 2007; Seixas et al., 2012; Sopher et al., 2011; Wilburn et al., 2011). HDL2 is a neurodegenerative autosomal dominant disorder that, like Huntington's disease (HD), is characterized by abnormal movements, dementia, and psychiatric syndromes (Margolis et al., 2005). Also like HD, HDL2 features prominent cortical and striatal atrophy and IC2-positive intranuclear inclusions that are presumably polyglutamine aggregates. HDL2 is caused by CTG/CAG repeat expansions at the *Junctophilin-3 (JPH3)* locus (Holmes et al., 2001). The CTG expansion is located within the alternatively spliced exon 2A of *JPH3*, which is not part of the main transcript that encodes JPH3, a protein that contributes to the formation of junctional membrane structures (Takeshima et al., 2000). On the sense strand, three splice variants that include exon 2a have been described, placing the CTG repeat in polyleucine or polyalanine open reading frames, or in the 3' UTR.

The pathogenic mechanisms underlying HDL2 are not completely known. Although CUG repeats may decrease *JPH3* mRNA, and thus JPH3 expression, it is unlikely that HDL2 results solely from loss of JPH3 function given that *JPH3* knockout mice exhibit motor impairments but no morphological changes in the brain (Nishi et al., 2002). Of note, expression of a *JPH3* transcript with expanded CUG repeats in cultured cells results in the formation of RNA foci that colocalize with MBNL1, as observed in HDL2 patients, and

causes cytotoxicity (Rudnicki et al., 2007). RNA foci formation may thus be one mechanism of disease in HDL2, but it does not account for the potential toxicity resulting from the presence of proteinaceous inclusions immunopositive for IC2. Despite the initial difficulty in finding evidence of a polyglutamine repeat expressed from the HDL2 locus, the existence of an antisense CAG transcript was eventually established through the development of transgenic mouse models of HDL2 in which an expanded CTG/CAG repeat in the human *JPH3* BAC was expressed (Wilburn et al., 2011). BAC-HDL2 mice recapitulate motor, neuropathological, and molecular phenotypes similar to those in HDL2 patients. The antisense CAG transcript is translated to produce a polyglutamine peptide, and BAC-HDL2 mice accumulate polyglutamine-containing, ubiquitin-positive nuclear inclusions in a pattern strikingly similar to those in patients. Of particular interest, it was shown that silencing of the CTG sense transcript does not prevent expression of the CAG antisense transcript and polyglutamine pathogenesis (Wilburn et al., 2011). These findings again highlight that the same repeat expansion can simultaneously cause toxicity resulting from aberrant RNA accumulation and expression of deleterious polyglutamine proteins.

The occurrence of bidirectional transcription across expanded repeats raises the possibility that complementary repeat RNAs form double-stranded structures. A recent study using a *Drosophila* model of DM1 revealed that toxicity resulting from the expression of expanded CTG transcripts is enhanced upon co-expression of expanded CAG transcripts. The co-expression of both transcripts leads to the generation of Dicer-2 and Argonaute-2-dependent 21mer triplet repeat-derived small interfering RNAs (siRNAs). The small RNAs produced in this fashion subsequently target CAG-containing genes, such as *Ataxin-2*, and influence their expression (Yu et al., 2011). Lawlor and colleagues have reported similar findings in two different *Drosophila* models. In one model, they fortuitously found that insertion of a CAG repeat trans-gene into the *cheerio* gene of *Drosophila* gives rise to both CAG and CUG-repeat containing transcripts, thus modeling bidirectional transcription across an expanded repeat disease loci. Because this phenomenon was accompanied by a progressive neurodegenerative phenotype, they set-out to model the pathogenic effects of complementary RNA repeat sequences independently of the *cheerio* gene. To this end, flies were made to express rCAG/rCUG_{~100} double-stranded RNA. These double-stranded expanded repeat RNAs were found to be significantly more toxic than either of the single-stranded RNA sequences and, once again, *Dicer* processing of the double-stranded RNA was essential for the observed pathology. Of interest, *Dicer* processing of double-stranded CAG/CUG repeats to form 21mers had a strong preference for (CAG)₇ production in this model. While the precise molecular mechanisms of Dicer-mediated toxicity in this model has yet to be elucidated, significant changes to the microRNA profile in rCAG/rCUG_{~100}-expressing neurons was observed (Lawlor et al., 2011). Taken together, these studies suggest that double-stranded RNAs formed upon bidirectional transcription of expanded repeats may contribute to the pathogenesis of expanded repeat diseases.

Repeat associated non-ATG translation

The previous section provides examples of microsatellite expansions that are bidirectionally transcribed, highlighting one of several pathogenic mechanisms possibly involved in repeat diseases. Recently, it was demonstrated that RNA transcripts with expanded CAG repeats can be translated in the absence of an ATG start codon (Zu et al., 2011). This mechanism, named Repeat Associated Non-ATG translation (RAN translation), occurs across expanded CAG repeats in all reading frames, thus generating homopolymeric proteins containing long tracts of polyglutamine, polyserine and polyalanine. Moreover, because anti-sense CUG transcripts also undergo RAN translation in all three frames, polyleucine, polycysteine and polyalanine tracts are produced. In this manner, up to nine potentially toxic entities may be generated: the two RNA transcripts containing expanded CAG or CUG trinucleotides

produced from bidirectional transcription, the polyglutamine protein expressed from AUG-initiated translation, and the six homopolymeric proteins produced from RAN translation (Pearson, 2011).

Through a series of elegant studies, Dr. Ranum's group has shown that RAN translation may depend on hairpin formation, and is dependent on repeat length, with longer repeat tracts being associated with simultaneous expression of multiple homopolymeric proteins (Zu et al., 2011). They also provide evidence that RAN translation products cause apoptosis in cultured cells and, of particular importance, they show that RAN translation occurs in disease-relevant tissues of SCA8 and DM1 patients with CAG/CTG expansions. To determine whether homopolymeric proteins are expressed *in vivo*, they developed peptide antibodies to putative RAN translated SCA8-polyalanine proteins and DM1-polyglutamine proteins. An accumulation of SCA8 polyalanine expansion proteins was found in cerebellar Purkinje cells in postmortem samples from SCA8 patients and in SCA8 BAC-EXP transgenic mice. DM1 polyglutamine expansion proteins accumulated as nuclear aggregates in DM1 mouse tissue and DM1 patient cardiac myocytes, leukocytes, and myoblasts (Zu et al., 2011). A schematic of RAN translation in DM1 is provided in Fig. 1B.

Based on studies of RAN translation, it is now clear that canonical rules of translation do not apply to CAG expansion tracts. Whether other trinucleotide repeats, and even tetranucleotide, pentanucleotide and hexanucleotide repeats, undergo RAN translation is most certainly under investigation, as are the consequences of RAN translated protein expression in disease.

Epigenetic changes and haploinsufficiency

Research focusing on repeat diseases shows that repeat expansions can impair gene transcription through epigenetic changes leading to DNA methylation and heterochromatin formation (Al-Mahdawi et al., 2008; Todd et al., 2010), as well as through aberrant binding of the transcriptional machinery (Cong et al., 2012; Nasrallah et al., 2012). It has also been demonstrated that non-coding expansions modify RNA and miRNA transcription, the latter responsible for regulating the expression of numerous targeted genes (Kelley et al., 2012; Renoux and Todd, 2012). Specifically, methylation of repeat sequences, as well as hypermethylation of neighboring islands rich in cytosine-phosphate-guanine (CpG) within promoter regions, lead to chromatin changes and gene silencing resulting in haploinsufficiency (Al-Mahdawi et al., 2008; Santoro et al., 2012). CpG dinucleotides are not uniformly distributed throughout the genome, but are concentrated in regions of repetitive genomic sequences characterized by a higher frequency of CpG sequences known as CpG islands (Caiafa and Zampieri, 2005). These islands are often located around promoters of active housekeeping genes and are not methylated. Conversely, CpG sequences in inactive genes are usually methylated to suppress their expression. When CpG islands are methylated, they attract methyl-CpG binding proteins, such as MeCP2, MBD1, and MBD2 that associate with a histone deacetylase complex. This results in the deacetylation of lysine residues in histones H3 and H4 and, consequently, modifies chromatin by compressing the shape of the genomic material and inactivates the gene (Baker and El-Osta, 2003). Of note, the position of methylation in the transcriptional unit is important; while methylation in the promoter region leads to gene silencing, methylation in the gene body stimulates transcription/elongation and may impact splicing (Jones, 2012). In fact, in mammals, the initiation of transcription, but not subsequent transcription after initiation, is sensitive to DNA methylation silencing. Moreover, misregulated bidirectional transcription levels have been demonstrated to have a direct influence on RNA-mediated epigenetic regulation and play an important role in several neurodegenerative diseases.

Histone modification and heterochromatin formation have been implicated in DM1 (Filippova et al., 2001; Steinbach et al., 1998), SCA8 (Chen et al., 2009), FRDA (Al-Mahdawi et al., 2008), and FXS (Todd et al., 2010). For example, premutations in the *FMR1* gene, which are characterized by 55–200 CGG repeats, result in enhanced *FMR1* mRNA levels, as well as the formation of RNA foci, yet FMRP protein expression remains relatively preserved (Todd et al., 2010). In contrast, full mutations (i.e. expansions of more than 200 CGG repeats) cause FXS as a consequence of methylation of the repeats and hypermethylation of neighboring CpG islands in the promotor region of *FMR1*. This altered methylation process prevents the binding of transcription factors and results in gene silencing (Fig. 1C). More specifically, allele silencing is associated with histone H3 dimethylation and trimethylation at lysine 9, histone H3 trimethylation at lysine 27, and histone H4 trimethylation at lysine 20, all changes similar to the mechanism involved in the formation of pericentric heterochromatin (Kumari and Usdin, 2010). Ultimately, *FMR1* silencing and the resulting absence of the FMRP protein cause FXS.

CCCTF-binding factor (CTCF) is a multifunctional regulator of several genomic processes, including subnuclear localization, long-range chromatin interaction, and imprinting (Bell and Felsenfeld, 2000; Hark et al., 2000; Splinter et al., 2006; Yusufzai et al., 2004). CTCF has also been shown to play a central role in transcription by associating with CTCF binding sites to mediate both transcription activation and repression (Filippova et al., 1996; Vostrov and Quitschke, 1997). Association of CTCF with CTCF binding sites is modulated by DNA methylation, which alters its regulatory function (Wang et al., 2012a). Moreover, CTCF has an important enhancer-blocking task when binding to specific sites in the promoter region, this way sealing the boundaries of adjacent genes (Filippova et al., 2001; Hou et al., 2008). Of interest, two CTCF binding sites flanking the microsatellite region in *DMPK* have been found to act as an insulator element between *DMPK* and *SIX5*. While the presence of a normal repeat length microsatellite combined with CTCF binding sites forms an efficient insulator unit, CpG islands located in the CTCF binding sites become hypermethylated in DM1 repeat expansion carriers. This prevents CTCF binding and CTCF-mediated insulator activity, and thus contributes to DM1 etiology (Steinbach et al., 1998). These studies suggest that repeat expansions can induce epigenetic changes leading to haploinsufficiency.

RNA-binding proteinopathies

As demonstrated by the above examples, proper RNA processing is required for normal cellular functions, and gene mutations that result in the accumulation of toxic RNA species cause several diseases. In most of the RNAopathies described, a consequence of RNA foci formation is the abnormal sequestration and loss of function of RNA-binding proteins, such as MBLN, emphasizing the involvement of RNA dysregulation in neurodegeneration. Indeed, mutations or abnormalities that directly affect RNA-binding proteins have detrimental consequences. RNA-binding proteins, which regulate various RNA-dependent functions, are crucial determinants of gene expression. They play a role in pre-mRNA splicing and maturation, as well as mRNA transport, storage, stability and translation (Gorospe, 2012). Among the RNA-binding proteins causative of disease are nuclear polyadenylate binding protein nuclear 1, zinc finger protein 9, survival of motor neuron protein (SMN), FMRP, TDP-43 and FUS (Table 1).

Loss and gain of function in RNA-proteinopathies

Deletion of the *survival of motor neuron 1 (SMN1)* gene causes spinal muscular atrophy (SMA), a disorder characterized by muscle weakness and atrophy resulting from lower motor neuron degeneration (Table 1). As its name aptly implies, *SMN1* encodes a protein that is important for the survival of motor neurons. The SMN protein, which exhibits RNA-binding activity, plays a critical role in the biogenesis of small nuclear ribonucleoprotein

particles (snRNPs), which are essential components of the spliceosomal machinery (Neuenkirchen et al., 2008). Severity of SMA corresponds to the degree of functional SMN protein deficiency (Lefebvre et al., 1997). Despite the fact that humans have two genes encoding SMN (*SMN1* and *SMN2*), *SMN2* cannot fully compensate for *SMN1* deletion. A single base pair substitution located in exon 7 of *SMN2* alters the splicing pattern of *SMN2* pre-mRNA, causing frequent exclusion of exon 7, and the production of a truncated and unstable SMN protein (Lorson and Androphy, 2000). As such, *SMN2* produces only about 10% of the full-length functional SMN protein compared to *SMN1*. Of interest, *in vivo* splicing assays demonstrated that the splicing factor hnRNP G regulates inclusion of *SMN2* exon 7 (Hofmann and Wirth, 2002); this RNA-binding protein is also sequestered in *FMR1* RNA foci (Sellier et al., 2010).

Given its central role in snRNP complex formation, decreases in SMN expression are expected to result in generalized defects in splicing across a range of transcripts. Indeed, SMN depletion in a mouse model of SMA, which recapitulates the main features of human disease, leads to decreased snRNP assembly activity in the spinal cord, brain and kidneys (Gabanella et al., 2007). The extent of reduction in snRNP assembly correlates with disease severity in SMA mice, and widespread pre-mRNA splicing defects in numerous transcripts of diverse genes are observed (Gabanella et al., 2007; Zhang et al., 2008). Given that aberrant splicing may be a significant contributor to motor neuron degeneration in SMA, a detailed assessment of the expression patterns of transcripts in motor neurons is warranted. In a similar fashion, many groups have undertaken studies to identify RNA targets for the RNA-binding proteins FMRP, TDP-43, and FUS (Colombrita et al., 2012; Darnell et al., 2005; Polymeridou et al., 2011; Tan et al., 2012; Tollervey et al., 2011; Xiao et al., 2011).

Whereas SMA results from loss of an RNA-binding protein, ALS and certain pathological subtypes of FTD are characterized by the abnormal aggregation of RNA-binding proteins. ALS and FTD are two devastating neurodegenerative diseases that, because of significant clinical, neuro-pathological and genetic overlap, are thought to be situated at points along one continuous clinicopathological spectrum of multi-system neurodegenerative diseases. ALS, the most frequent of motor neuron diseases, is characterized by the degeneration of upper and lower motor neurons leading to muscle weakness, spasticity and atrophy, with patients having a mean life expectancy of three to five years after disease onset (Bradley, 2000; Johnston et al., 2006). FTD, the second-most common cause of early onset dementia after Alzheimer's disease, is characterized by neuronal degeneration in the frontal and temporal lobes, which leads to progressive deterioration in behavior, personality and/or language (Graff-Radford and Woodruff, 2007; Mercy et al., 2008).

As with many neurodegenerative diseases, a pathological hallmark of ALS and FTD is the presence of abnormal ubiquitin-immunopositive inclusions within neurons and glia. With respect to the protein undergoing aggregation, FTD is broadly divided into cases with inclusions immunopositive for tau (FTLD-tau), TDP-43 (FTLD-TDP-43), or FUS (FTLD-FUS). Approximately half of FTD cases have tau pathology, 40–45% have TDP-43 pathology, and 5–10% have FUS pathology (Halliday et al., 2012). In ALS, TDP-43 inclusions are a consistent feature in the majority of sporadic cases (Neumann et al., 2006), and are present in familial ALS cases caused by mutations in *TARDBP*, the gene encoding TDP-43 (Van Deerlin et al., 2008). Over 40 *TARDBP* mutations have been identified in sporadic and familial ALS patients, and in rare FTD patients, with most mutations lying in the C-terminal glycine-rich region of TDP-43 (Da Cruz and Cleveland, 2011). *TARDBP* mutations occur in approximately 5% of familial ALS cases and 2% of sporadic cases. Likewise, over 40 *FUS* mutations have been identified in sporadic and familial ALS patients, and in rare FTD patients, and they too are mostly located in the C-terminus of the protein (Da Cruz and Cleveland, 2011). Overall, coding and splicing mutations in *FUS* are

now estimated to cause about 4% of familial ALS and 1% of sporadic ALS. Of interest, a recent study identified *FUS* mutations in patients affected with hereditary essential tremor 4 (ETM4), a neurodegenerative disorder characterized by postural and motion tremor (Table 1) (Merner et al., 2012). A nonsense mutation located in the first half of the *FUS* gene was shown to lead to an RNA loss-of-function due to nonsense-mediated decay. This differs from mutant *FUS* transcripts in ALS, which do not undergo such degradation.

That mutations in *TARDBP* and *FUS* cause ALS provides evidence of a direct link between abnormalities in these RNA-binding proteins and neurodegeneration. In fact, TDP-43 and FUS pathology are not restricted to ALS and FTD. TDP-43 inclusions are observed in a variety of neurodegenerative disorders, including different types of dementias, myopathies, polyglutamine diseases, and parkinsonism (Lagier-Tourenne et al., 2010), whereas FUS pathology is found in a wide spectrum of polyglutamine diseases, such as Huntington's disease and spinocerebellar ataxias (Doi et al., 2010; Woulfe et al., 2010).

TDP-43- and FUS-mediated neuronal death is likely caused via multiple pathways involving a combination of toxic gains and loss of function. TDP-43 and FUS are both hnRNP family members that contain RNA-binding motifs, directly bind to RNA in addition to single and double-stranded DNA, and are implicated in various steps of gene expression regulation, such as transcription, RNA splicing, RNA transport, and translation (Buratti and Baralle, 2008; Janknecht, 2005). Thus, loss of function of TDP-43 and FUS could have innumerable implications. Given that TDP-43 pathology is more prevalent than that of FUS in ALS and FTD, the subsequent section will focus specifically on TDP-43. For a more in-depth account of FUS, readers are referred to a review that highlights recent advances made in understanding the functions of FUS and the findings emerging from FUS animal models (Lanson and Pandey, 2012).

TDP-43 proteinopathies

In TDP-43 proteinopathies, the presence of cytoplasmic TDP-43 inclusions is associated with loss of nuclear TDP-43 (Arai et al., 2006; Neumann et al., 2006), and this redistribution appears to be an early event, at least in ALS (Giordana et al., 2010). The sequestration of TDP-43 from the nucleus to cytoplasmic inclusions is expected to result in a loss of TDP-43 function. Moreover, the inclusions themselves may be neurotoxic, merely inert by-products, or a protective mechanism used by cells to sequester harmful TDP-43 species. In addition to altered intracellular localization and aggregation, TDP-43 is phosphorylated and cleaved into C-terminal TDP-43 fragments (Arai et al., 2006; Neumann et al., 2006). These abnormal post-translational modifications may bestow toxic properties upon TDP-43, enhance TDP-43 aggregation, and/or cause TDP-43 dysfunction. For instance, phosphorylation of C-terminal TDP-43 fragments has been shown to render TDP-43 resistant to degradation and enhances its accumulation into insoluble inclusions (Zhang et al., 2010).

While the exact functions of TDP-43 have yet to be definitively determined, several groups have undertaken the daunting task of identifying TDP-43 RNA targets in cultured cells, mouse brain, and human brain from FTLTDP or ALS patients (Polymenidou et al., 2011; Tollervey et al., 2011; Xiao et al., 2011). These studies emphasize that TDP-43 interacts with a diverse spectrum of RNA targets with important functions in the brain. As such, a greater understanding of the role played by TDP-43 in regulating the splicing and expression of RNA is crucial. This information will help decipher how the characteristic traits of ALS and FTLTDP, such as TDP-43 mislocalization, truncation, and phosphorylation, influence TDP-43-regulated RNA metabolism. Moreover, an important goal will be to determine whether the function of proteins encoded by key RNA targets is altered in neuronal cells of

patients. This could provide valuable insight into the mechanisms of TDP-43 toxicity and eventually guide the development of therapeutics for both FTLTDP and ALS.

It must also be kept in mind that, although TDP-43 is largely a nuclear protein, it does shuttle between the nucleus and cytoplasm in a transcription-dependent manner, and a small proportion of TDP-43 is present in the cytoplasm under physiological conditions (Ayala et al., 2008; Winton et al., 2008). This suggests that, in addition to its nuclear functions, TDP-43 likely plays an important role in the cytoplasm. While its functions in the cytoplasm are not completely understood, it is noteworthy that the post-synaptic localization of TDP-43, in the form of RNA granules, is enhanced following depolarization of primary hippocampal neurons (Wang et al., 2008). Furthermore, studies utilizing primary hippocampal neurons from wild-type and TDP-43 transgenic mice reveal that TDP-43 regulates spinogenesis, an integral part of learning and memory (Majumder et al., 2012). TDP-43 overexpression suppresses dendritic spine development in primary hippocampal neurons and *in vivo*. Conversely, depletion of TDP-43 increases the number of spines in cultured hippocampal neurons through its actions on the Rac1-AMPA receptor pathway. Evidence also suggests that TDP-43 plays a role in helping cells withstand stressful conditions given that it relocates to cytoplasmic stress granules in response to harmful stimuli in cell and animal models (Colombrita et al., 2009; Dewey et al., 2011; Freibaum et al., 2010; Liu-Yesucevitz et al., 2010; McDonald et al., 2011; Moisse et al., 2009a,b). In addition to cytoplasmic stress granule formation, nuclear TDP-43 upregulation has recently been shown to provide protection to primary neurons against glutamate-induced excitotoxicity, a pathological process implicated in ALS (Zheng et al., 2012). Taken together, these findings suggest that TDP-43 regulates synaptic plasticity by controlling the transport and splicing of synaptic mRNAs, by modulating spinogenesis, as well as assisting in the physiological response to neuronal injury. It is therefore possible that mis-metabolism of TDP-43, as occurs in disease, would lead to impairments in hippocampal plasticity and render neurons more vulnerable to cellular stressors.

To gain a better understanding of the roles of TDP-43 in the central nervous system, and to gain insight on the mechanisms by which TDP-43 contributes to the development and progression of neurodegeneration, several groups have developed and characterized rodent TDP-43 transgenic models. Despite differences among the many TDP-43 transgenic models now established, which include variations in the regional expression of exogenous TDP-43, and the form of TDP-43 being expressed (i.e. mouse vs. human, wild-type vs. mutant, full-length vs. cleaved TDP-43), many commonalities exist among them [for a complete review, please see (Gendron and Petrucelli, 2011; Tsao et al., 2012; Wegorzewska and Baloh, 2011)]. For instance, ubiquitin accumulation, TDP-43 fragmentation, astrogliosis, microgliosis, axonal degeneration, mitochondrial abnormalities, neuronal loss, motor function impairments, and shortened lifespan are observed in many of the transgenic animals. It should be noted, however, that a recent study examining the effects of human TDP-43 overexpression in mice found that neuronal sensitivity to TDP-43 is dependent on the timing of its overexpression (Cannon et al., 2012). The phenotypes of mice differed depending on whether TDP-43 overexpression was initiated during early neuronal development, or instead was delayed until post-natal day 21. This study highlights that timing of TDP-43 overexpression in transgenic rodents must be considered when distinguishing normal roles of TDP-43, particularly as they relate to development, from its pathogenic roles in mature neurons.

In the majority of models, the presence of overt TDP-43 cytoplasmic inclusions is conspicuously lacking (Gendron and Petrucelli, 2011). TDP-43-immunopositive cytoplasmic inclusions are seldom observed when immunostaining of brain or spinal cord sections is carried out using phosphorylation-independent TDP-43 antibodies. However,

when using antibodies that detect TDP-43 phosphorylated at S403/S404 or S409/S410, sites abnormally phosphorylated in ALS and FTL-D-TDP (Hasegawa et al., 2008), TDP-43-positive intranuclear inclusions and punctate cytoplasmic inclusions are detected in many models, albeit with varying prevalence (Gendron and Petrucelli, 2011). Compared to phospho-TDP-43 immunoreactivity, increased ubiquitin expression, either in the form of multiple small aggregates, large aggregates, or present diffusely, is much more prevalent. Elevated ubiquitin expression, however, is not necessarily indicative of increased levels of ubiquitinated TDP-43 (Swarup et al., 2011; Xu et al., 2010). Even in mouse models in which cytoplasmic TDP-43 inclusions are present and ubiquitin immunoreactivity co-localizes with these inclusions, immunoprecipitation studies show that TDP-43 itself is not ubiquitinated (Swarup et al., 2011). These results suggest that enhanced TDP-43 expression in mice leads to the accumulation of yet-to-be identified ubiquitinated proteins.

The general scarcity of TDP-43 cytoplasmic inclusions within neurons of most TDP-43 transgenic animals would argue against a primary role for such inclusions in mediating the neurotoxic effects resulting from TDP-43 overexpression. Nonetheless, it does not exclude their pathogenic potential in ALS and FTL-D-TDP. Studies in yeast have shown that only those TDP-43 species that form aggregates are toxic, and that ALS-linked mutations that accelerate TDP-43 aggregation *in vitro* promote toxicity (Johnson et al., 2008, 2009). In differentiated human neuroblastoma cells, the expression and aggregation of a C-terminal TDP-43 fragment corresponding to caspase-cleaved TDP-43 is associated with increased cytotoxicity. This cytotoxicity likely results from a toxic gain of function since neither the nuclear distribution nor function of endogenous TDP-43 are altered in this model (Zhang et al., 2009). Of particular interest, Wang and colleagues have demonstrated that rapamycin, an activator of autophagy, alleviates learning and memory impairments, as well as loss of motor function, in a transgenic mouse model of FTL-D-TDP (Wang et al., 2012b). These behavioral improvements are accompanied by a reduction in forebrain neuron loss, a decreased number of cells bearing cytosolic TDP-43 inclusions, and reduced levels of insoluble, but not soluble, full-length TDP-43 and TDP-43 cleavage products. These findings suggest that rapamycin provides protection by blocking the formation of TDP-43 inclusions as a result of its capacity to enhance autophagic clearance of abnormally generated TDP-43 species before they aggregate in the cytoplasm.

While the above-mentioned studies are suggestive that TDP-43 inclusions are toxic, it remains challenging to distinguish whether the inclusions themselves are harmful, or whether the cytosolic TDP-43 species that eventually form aggregates are the toxic culprit. For instance, staurosporine-induced caspase 3 activation in cultured cells leads to the cleavage of full-length TDP-43 into ~25 and ~35 kDa fragments, and causes the redistribution of TDP-43 to the cytoplasm (Zhang et al., 2007). As mentioned above, caspase-cleaved TDP-43 is aggregation-prone and cytotoxic (Zhang et al., 2009); yet, whether this toxicity results expressly from the formation of TDP-43 inclusions is unclear. Compelling evidence that the cytosolic localization of TDP-43, and not its aggregation, causes neurotoxicity is provided by Barmada and colleagues. While EGFP-TDP-43^{A315T} overexpression in primary rat cortical neurons leads to the formation of detergent-resistant inclusions, it was found that neuronal toxicity resulting from EGFP-TDP-43^{A315T} expression occurs independently of inclusion formation. Rather, the amount of cytosolic EGFP-TDP-43^{A315T} is a powerful predictor of cell death, suggesting that soluble forms of cytosolic TDP-43 are neurotoxic (Barmada et al., 2010). In a similar fashion, Caccamo and colleagues found that the overexpression of a cytosolic C-terminal TDP-43 fragment (termed TDP-25) in mice is sufficient to cause cognitive deficits, yet neither inclusion formation nor nuclear depletion of TDP-43 are necessary for the onset of these behavioral alterations. They found that the earliest detectable cognitive deficits in their TDP-25 transgenic mice occurs at 6 months of age, at which point the only obvious change in the

brain is an increase in soluble TDP-25 levels (Caccamo et al., 2012). These findings suggest that the cleavage of TDP-43 conveys a toxic gain of function via the formation of cytosolic C-terminal TDP-43 fragments.

It must be mentioned, however, that C-terminal TDP-43 fragments are formed in many, but not all, TDP-43 transgenic rodent models (Gendron and Petrucelli, 2011). In some transgenic mouse models, there exists a correlation between the appearance of C-terminal TDP-43 fragments of approximately ~25 kDa and disease progression (Stallings et al., 2010; Wegorzewska et al., 2009; Wils et al., 2010). While correlative, these findings are consistent with the hypothesis that ~25 kDa C-terminal fragments play an important role in TDP-43-mediated neurodegeneration. Arguing against this hypothesis are findings from other TDP-43 transgenic models that suggest TDP-43 fragments are generated only after the first appearance of symptoms (Zhou et al., 2010), or are not generated at all (Igaz et al., 2011; Shan et al., 2010). Although this indicates that, at least in some rodent models, TDP-43 cleavage products are not responsible for TDP-43-mediated toxicity, it does not rule out the potentially harmful consequence of TDP-43 cleavage in ALS and FTLTDP. Unlike transgenic rodent models of TDP-43 overexpression, cleavage of TDP-43 in human neurons would presumably deplete the pool of functional, full-length TDP-43. Likewise, while TDP-43 inclusions themselves may, or may not, be toxic entities, the fact that they result in the depletion of TDP-43 from the nucleus undoubtedly has negative effects on TDP-43 function.

In ALS and FTLTDP, a loss of nuclear TDP-43 staining is commonly observed in affected spinal cord and/or brain neurons (Neumann et al., 2006). While exogenous TDP-43 in TDP-43 transgenic rodents is primarily nuclear, in some models, a loss of nuclear TDP-43 in selectively vulnerable neurons is observed prior to obvious signs of degeneration (Igaz et al., 2011; Tsai et al., 2010; Wegorzewska et al., 2009; Wils et al., 2010). For example, Igaz and colleagues found that the rate and extent of neurodegeneration in transgenic mice that inducibly overexpress human TDP-43 with a mutated nuclear localization signal (iTDP-43 NLS) correlate with loss of endogenous nuclear mouse TDP-43 (mTDP-43). The reduction in mTDP-43 resulted from decreased mTDP-43 mRNA and protein expression. TDP-43 auto-regulates its mRNA levels through a negative feedback loop (Ayala et al., 2011); as such, a common feature in transgenic mice overexpressing human TDP-43 is the down-regulation of mTDP-43 (Xu et al., 2010, 2011). Igaz and colleagues have thus speculated that perturbations of endogenous nuclear mTDP-43 in transgenic mice results in loss of normal mTDP-43 function, culminating in the degeneration of selectively vulnerable neurons. Nonetheless, it remains possible that loss of mTDP-43 is not responsible for the phenotypes observed in TDP-43 transgenic mice given that transgenic mice overexpressing mTDP-43 display similar neurodegeneration and share many characteristics with mice overexpressing human TDP-43, even though they maintain high levels of nuclear mTDP-43 (Tsai et al., 2010). Furthermore, mouse and human TDP-43 are highly homologous, especially within their RNA recognition motifs (Wang et al., 2004).

While the contribution of decreased endogenous mTDP-43 to the development of phenotypes in TDP-43 transgenic rodents remains enigmatic, there is a great deal of evidence supporting that TDP-43 is critical to the survival of mice. Knocking-out mTDP-43 expression results in embryonic lethality (Chiang et al., 2010; Kraemer et al., 2010; Sephton et al., 2010; Wu et al., 2010). Furthermore, even if embryonic lethality is overcome by knocking-out mTDP-43 expression after the birth of mice, mice exhibit a dramatic loss of body fat followed by rapid death (Chiang et al., 2010). Wu and colleagues thus undertook a different approach to investigate the consequences of mTDP-43 loss of function *in vivo* and found that targeted depletion of mTDP-43 in spinal cord motor neurons of mice leads to the progressive, male-dominant, development of ALS-related phenotypes, including kyphosis,

motor dysfunction, muscle weakness and atrophy, motor neuron loss, and gliosis. Moreover, these mice displayed enhanced levels of ubiquitin, and ubiquitinated-proteins, in spinal cord motor neurons depleted of mTDP-43 (Wu et al., 2012). These findings demonstrate an essential role of TDP-43 in the survival and function of spinal cord motor neurons. They also suggest that loss of TDP-43 may cause impairment of the proteasome and/or autophagy systems, leading to the accumulation of ubiquitinated proteins in diseased cells. Of relevance to this particular point, it has been shown that TDP-43 plays a role in regulating autophagy in cultured cells (Bose et al., 2011). Upon depletion of TDP-43, levels of mRNA encoding the major autophagy component, Atg7, are decreased, consequently impairing autophagy and leading to the accumulation of ubiquitinated proteins.

While the mechanisms driving TDP-43-mediated toxicity in ALS and FTLTDP have yet to be definitively elucidated, great strides have been made, and will surely continue to be made, to improve our understanding of TDP-43 function and dysfunction.

C9ORF72-linked ALS and FTD

In 2006 and 2007, genetic linkage to a locus on chromosome 9p was established for several families with a combination of FTD and ALS (Morita et al., 2006; Valdmanis et al., 2007; Vance et al., 2006). Following years of intense investigation by geneticists worldwide, two independent groups identified an expanded hexanucleotide (GGGGCC) repeat in a noncoding region of the *C9ORF72* gene as the causative mutation for FTD/ALS-linked to the chromosome 9p locus (DeJesus-Hernandez et al., 2011; Renton et al., 2011). While mutation frequency varies among populations of c9FTD/ALS patients, the average mutation frequencies reported in North American and European populations are 37% for familial ALS, 6% for sporadic ALS, 21% for familial FTD, and 6% for sporadic FTD (Rademakers et al., 2012). This renders mutations in *C9ORF72* the most common genetic cause of FTD and ALS, thus eliciting tremendous excitement in the field and a flurry of efforts aims at deciphering how these repeat expansions cause disease.

Despite clinical heterogeneity, *C9ORF72* expanded repeat carriers often develop a combination of frontotemporal lobar dysfunction and motor abnormalities (Hsiung et al., 2012), and there exists an association between *C9ORF72* mutation and psychosis (Snowden et al., 2012). c9FTD/ALS cases show “classical” TDP-43 inclusions in many neuroanatomical regions, including the extramotor cerebral cortex, hippocampus, basal ganglia, substantia nigra, and lower motor neurons of the brainstem and spinal cord (Murray et al., 2011). This suggests that TDP-43-mediated neurodegeneration contributes to the development of disease, as in sporadic ALS and FTLTDP. Furthermore, atypical ubiquitin/p62-positive, but TDP-43-negative, neuronal inclusions within cerebellar granules cells and hippocampal pyramidal neurons are observed (Al-Sarraj et al., 2011; Boxer et al., 2011; Murray et al., 2011). This unique and highly characteristic feature suggests that *C9ORF72* mutations cause the abnormal accumulation of yet-to-be identified ubiquitinated proteins. The involvement of these proteinaceous aggregates in disease pathogenesis has yet to be elucidated.

Using a semi-quantitative, repeat-primed PCR method, it has been estimated that control subjects have up to 23 GGGGCC repeats, while c9FTD/ALS patients carry between 700 and 1,600 repetitions (DeJesus-Hernandez et al., 2011). Nonetheless, the minimal repeat size necessary for disease to manifest may be much lower. It should also be pointed out that there is evidence of somatic instability of the *C9ORF72* repeat (DeJesus-Hernandez et al., 2011); as such, repeat lengths may vary among different tissues within the same individual, potentially confounding the accurate determination of repeat lengths.

At present, the functions of C9ORF72 protein remain completely unknown. Through alternative splicing, at least three distinct transcripts are produced from the human *C9ORF72* gene, and these are predicted to encode two protein isoforms. Transcript variants 2 and 3 lead to the expression of a 481 aa protein (isoform A) encoded by exons 2–11, whereas variant 1 leads to the expression of a 222 aa protein (isoform B) encoded by exons 2–5. The GGGGCC repeat is located between two alternatively spliced noncoding first exons (exons 1a and 1b) of *C9ORF72*. Depending on their use, the expanded repeat is located either in intron 1 (variants 1 and 3) or the promoter region (variant 2). Through RT-PCR, DeJesus-Hernandez and colleagues found that these three *C9ORF72* transcripts are expressed in a variety of tissues. To address whether expanded repeats affect *C9ORF72* expression in a transcript-specific manner, they examined expression of each of the three *C9ORF72* mRNA transcripts in frontal cortex tissue and lymphoblast cell lines from GGGGCC-repeat carriers. They found an absence of variant 2 expression from the mutant RNA allele, leading to a significant overall reduction in *C9ORF72* transcripts encoding C9ORF72 isoform A. In a similar fashion, preliminary data from Renton and colleagues suggests that mRNA expression of *C9ORF72* transcript variant 2 is decreased in frontal cortex of mutation carriers compared to normal controls. Of interest, another group found that the expression of the three *C9ORF72* transcripts was decreased in the frontal cortex of *C9ORF72* mutations carriers from a Flanders-Belgium cohort of ALS/FTD patients (Gijssels et al., 2012). While these studies must be replicated using a larger number of samples, it remains possible that c9FTD/ALS may result, at least in part, from a loss of C9ORF72 function. Unfortunately, studies aimed at evaluating C9ORF72 protein expression between controls and expanded repeat carriers have been greatly hindered due to the lack of specific, sensitive and well-characterized C9ORF72 antibodies. Studies to date have either shown a decrease in C9ORF72 protein expression in fibro-blast cell lines from ALS patients (Renton et al., 2011), or have failed to detect marked changes in C9ORF72 expression in brain tissue from expanded repeat carriers (Cooper-Knock et al., 2012; DeJesus-Hernandez et al., 2011; Hsiung et al., 2012; Simon-Sanchez et al., 2012; Snowden et al., 2012; Stewart et al., 2012). Cellular and animal models of *C9ORF72* knock-down or knock-out will prove valuable in determining whether *C9ORF72* haploinsufficiency is a pathological mechanism of disease.

While the contribution of altered C9ORF72 function in c9FTD/ALS has yet to be established, a toxic RNA gain-of-function is a likely mechanism of disease. The Rademakers group has shown that transcripts containing the GGGGCC repeat accumulate as nuclear RNA foci in the frontal cortex and spinal cord of *C9ORF72* mutation carriers (Fig. 1D). As in other noncoding expansion disorders, these RNA foci will likely alter the function of one or more RNA-binding proteins, resulting in downstream changes in alternative splicing and gene expression. The race is on to generate *in vivo* models for the expression of human *C9ORF72* containing expanded repeats, to determine which RNA-binding proteins are sequestered by RNA foci, and to identify the RNA targets abnormally expressed in *C9ORF72* mutation carriers.

In addition to TDP-43-mediated toxicity, loss of C9ORF72 function, and RNA-toxicity resulting from foci formation, various other mechanisms may contribute to disease. These include bidirectional transcription and RAN translation of *C9ORF72* repeat expansions, as well as epigenetic changes. Future studies on c9FTD/ALS will surely be inspired by previous findings related to neurological disorders displaying similar etiologies or pathologies. At present, it would appear as though we have merely scratched the surface in our quest to identify and understand the mechanisms of disease involved in c9FTD/ALS.

Concluding remarks

Aberrant functions of RNA and RNA-binding proteins are recurrent themes in neurodegeneration, underscoring the importance of precise RNA metabolism for neuronal survival. Lessons learned from research on each disease have greatly expanded our knowledge for a wide spectrum of neurological disorders. Because of this, it is becoming increasingly apparent that multiple pathogenic mechanisms can act independently or co-exist in neurodegenerative diseases, especially those caused by micro-satellite repeat expansions. For instance, bidirectional transcription and RAN translation both occur in SCA8 and DM1.

At present, nuclear RNA foci formation and RNA-binding protein sequestration are observed in approximately nine neurodegenerative disorders (Table 1). The sequestration of RNA-binding proteins, which play important RNA regulatory roles, is expected to subsequently alter the function of their numerous RNA targets. Of interest, certain neurological diseases caused by different gene mutations are characterized by the sequestration of the same RNA-binding proteins. Likewise, cytoplasmic inclusions of the same protein, such as TDP-43, are found in various neurodegenerative disorders. With regards to c9FTD/ALS, the co-occurrence of nuclear RNA foci and cytoplasmic proteinaceous inclusions could suggest either the involvement of two parallel pathological processes, or that RNA foci formation indirectly influences extra-nuclear functions that result in inclusion formation.

Also of interest is the reported mosaicism in repeat expansion carriers. Genotypic variations in repeat expansion length among patients may lead to different RNA expression outcomes, consequently explaining the discrepancies in phenotype among members of familial syndromes. In fact, genotypic variations in expansion length have been associated to different diseases. For example, intermediate repeat expansion length in *FMR1* results in FXTAS, while long expansions give rise to FXS (Ladd et al., 2007). Similarly, intermediate repeat expansion length in *ATXN2* is associated with ALS and Parkinson's disease (Elden et al., 2010; Wang et al., 2009), while longer expansions cause spinocerebellar ataxia type 2 (Pulst et al., 1996); and intermediate repeat expansion length in *ATXN8OS* is associated with several neurodegenerative diseases, including Parkinson's disease and different subtypes of ataxia (Sulek et al., 2003; Wu et al., 2004), while longer repeat expansions cause SCA8 (Moseley et al., 2006). One could thus speculate that intermediate repeat lengths in *C9ORF72* may eventually be associated to neurodegenerative diseases other than ALS and FTD.

While our understanding of RNA-mediated mechanisms of neuro-toxicity grows, it must be kept in mind that RNA processes should not be studied in isolation of environmental influences. Rather, the possibility of toxic entities triggering epigenetic changes in patients suffering from neurodegenerative diseases should be considered, as such changes could contribute to phenotype variations. Given that DNA methylation is a reversible epigenetic modification, and given that demethylating agents are being developed for cancer treatment (Hofmann, 2011), DNA-demethylating agents in combination with HDAC inhibitors could be promising drugs for patients suffering from certain RNAopathies. In this same vein, as progress in therapeutic development is made for some neurodegenerative diseases, investigators of lesser understood neurological disorders are likely to benefit by using the information to guide treatment strategies. For instance, it has been proposed that the best way to treat RNA toxicity in DM1 is to rid cells of the "poisonous" culprit, namely the mutated mRNA (Mahadevan, 2012). This strategy is being actively pursued using antisense oligonucleotides and gene therapy vectors expressing antisense RNAs (Francois et al., 2011; Lee et al., 2012), and could have wide-reaching implications for many RNA-dominant

disorders in which expanded repeats are expressed. Nonetheless, since different mechanisms may be implicated in disease onset and progression, these may need to be therapeutically targeted in a timed or concerted fashion. For example, in the case of c9FTD/ALS, TDP-43-based treatments may have to be paired with additional therapeutic strategies aimed at alleviating the pathogenic consequences of *C9ORF72* expanded repeats.

Overall, this is an exciting time for research aimed at elucidating the pathogenic mechanisms of RNAopathies and RNA-binding proteinopathies. Past discoveries are expected to form a sound basis for future studies, as well as inspire innovative avenues of investigation. As our understanding of disease mechanisms grows, so too will the probability of developing effective therapeutic strategies.

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Fig. 1.

Aberrant RNA mechanisms in neurodegeneration. (A) Bidirectional Transcription: schematic representation of *ATXN8* and *ATXN8OS* with repeat expansions located in the open reading frame (ORF) or the 3' UTR region, respectively. The CAG/CTG trinucleotide expansions are transcribed from the sense and antisense strands, consequently encoding for polyalanine and polyglutamine tracts in SCA8 patients. (B) Repeat Associated Non-ATG translation (RAN translation): schematic representation of mutant *DMPK* in DM1. The repeat expansion is transcribed bidirectionally, and translation can be initiated in any reading frame of the CUG/CAG repeat expansion, consequently encoding for polyleucine, polycysteine, polyalanine, polyglutamine, and polyserine tracts. (C) Epigenetic changes leading to haploinsufficiency: schematic representation of *FMR1* in FXS patients. (CGG)₂₀₀ repeats are methylated and CpG islands encompassing the region are hypermethylated. This leads to histones H3 and H4 deacetylation and chromatin modification, consequently compressing the shape of the genomic material. Binding of methyl (CH₃) groups activates histone deacetylase complex, leading to gene silencing and haploinsufficiency. (D) RNA foci formation: schematic representation of the three transcripts and two protein isoforms of *C9ORF72*. Expanded GGGGCC repeats in *C9ORF72* lead to decreased expression of transcript 2, and potentially RNA foci formation by transcripts 1 and 3. TDP-43 pathology is also present in *C9ORF72* repeat expansion carriers affected with ALS and FTLTDP.

Table 1

Summary of neurodegenerative RNA pathologies.

Gene	Disease	Mutation	Location	Mechanism	Process	References
<i>SPG7 (paraplegin)</i>	HSP7 (Hereditary spastic paraplegia 7)	Missense, nonsense, rearrangement, in/del, splicing	Exons	RNA loss of function	Protein accumulation caused by non-functional m-AAA protease, aberrant ribosome biogenesis	(Casari et al., 1998) (Koppen et al., 2007)
Coding	<i>GARS (glycyl tRNA synthetase)</i>	DSMAVA (Distal spinal muscular atrophy type V)	Missense	Exons	unknown	Aberrant translation (Antonellis et al., 2003)
	<i>CMT2D (Charcot Marie Tooth disease type 2D)</i>	CMT2D	Missense	Exons	unknown	Aberrant translation (Antonellis et al., 2003)
	<i>YARS (tyrosyl tRNA synthetase)</i>	DI-CMT (Dominant intermediate Charcot Marie Tooth disease)	Missense, in/del	Exons	unknown	Aberrant translation (Jordanova et al., 2006)
Non-coding	<i>DMPK (dystrophia myotonica protein kinase)</i>	DM1 (Myotonic dystrophy type 1)	Expanded (CTG) _{50-5,000}	3 UTR	RNA gain of function, aberrant splicing	RNA foci sequestering MBLN, chromatin changes (Fu et al., 1992; Mahadevan et al., 1992) (Jiang et al., 2004) (Thornton et al., 1997)
	<i>ATXN8OS (non-protein coding ataxin 8 opposite strand)</i>	SCA8 (Spinocerebellar ataxia type 8)	Expanded (CTG) ₁₀₇₋₁₂₇	3 UTR	RNA gain of function, aberrant splicing	RNA foci sequestering MBLN, histone modification (Koob et al., 1999b) (Daughters et al., 2009) (Chen et al., 2009)
	<i>E46L (neuron survival protein ataxin 10)</i>	SCA10 (Spinocerebellar ataxia type 10)	Expanded (ATTCT) _{280-4,500}	Intron 9	RNA gain of function	RNA foci sequestering hnRNP K (Matsuura et al., 2000) (White et al., 2010)
Non-coding	<i>BEAN (brain-expressed associated with NEDD4)</i>	SCA31 (Spinocerebellar ataxia type 31)	Inserted (TGGAA) ₅₀₀₋₇₆₀	Intron	RNA gain of function	RNA foci sequestering SFRS1 & SFRS9, chromatin changes (Sato et al., 2009)
	<i>FXN (frataxin)</i>	FRDA (Friedreich ataxia)	Expanded (GAA) _{210-1,186}	Intron 1	RNA loss of function	chromatin changes (Campuzano et al., 1996) (Greene et al., 2007)

Gene	Disease	Mutation	Location	Mechanism	Process	References
JPH3 (<i>junctionophilin-3</i>)	HDL2 (Huntington disease-like 2)	Expanded (CTG) ⁴¹	3 UTR	RNA gain of function, aberrant splicing	RNA foci sequestering MBNL	(Holmes et al., 2001) (Rudnicki et al., 2007)
C9ORF72 (<i>chromosome 9 open reading frame 72</i>)	ALS (Amyotrophic lateral sclerosis)	Expanded (GGGGCC) _{250-1,600}	Promotor + intron 1	Unknown	RNA foci	(Dejesus-Hernandez et al., 2011; Renton et al., 2011)
	FTLD (Frontotemporal lobar degeneration)	Expanded (GGGGCC) _{250-1,600}	Promotor + intron 1	Unknown	RNA foci	(Dejesus-Hernandez et al., 2011; Renton et al., 2011)
ZNF9 (<i>zinc finger protein 9</i>)	DM2 (Myotonic dystrophy type 2)	Expanded (CCTG) _{75-11,000}	Intron 1	RNA gain of function, aberrant splicing	RNA foci sequestering MBNL	(Liquori et al., 2001) (Mankodi et al., 2001)
FMR1 (<i>fragile X mental retardation protein</i>)	FXTAS (Fragile X-associated tremor/ataxia syndrome)	Expanded (CGG) ₅₅₋₂₀₀	5 UTR	RNA gain of function, aberrant splicing	RNA foci sequestering MBNL, hnRNP G, hnRNP A2/B1, SAM68, Pur, lamin A/C, chromatin changes	(Hagerman et al., 2001) (Iwahashi et al., 2006) (Sellier et al., 2010)
	FXS (Fragile X syndrome)	Expanded (CGG) ₂₀₀	5 UTR	RNA loss of function	Chromatin changes	(Kremer et al., 1991) (Naumann et al., 2009)
PABPN1 (<i>polyadenylate binding protein nuclear 1</i>)	OPMD (Oculopharyngeal muscular dystrophy)	Expanded (GCC) ₁₂₋₁₇	Exon 1	RNA gain of function	PABPN1 protein aggregates, disruption of cellular pre-mRNA metabolism	(Brais et al., 1998) (Calado et al., 2000)
SMN 1 (<i>survival of motor neuron 1</i>)	SMA (Spinal muscular atrophy)	Missense, in/del, inversion, gene deletion	Exons	RNA loss of function	No coding gene, decrease of small ribonucleoprotein assembly	(Lefebvre et al., 1995) (Gabanella et al., 2007)
SMN 2 (<i>survival of motor neuron 2</i>)	SMA (Spinal muscular atrophy)	Silent	Exons	Aberrant splicing	Decrease of small ribonucleoprotein assembly	(Hahnen et al., 1996) (Schwartz et al., 1997) (Gabanella et al., 2007)

RNA-binding

Gene	Disease	Mutation	Location	Mechanism	Process	References
<i>TARDPB</i> (tar DNA-binding protein)	ALS (Amyotrophic lateral sclerosis)	Missense, nonsense, in/del, splicing	Exons	unknown	TDP-43 protein aggregates	(Kabashi et al., 2008) (Sreedharan et al., 2008) (Kabashi et al., 2010)
		Missense	Exons, splice sites	Unknown	TDP-43 protein aggregates	(Benajiba et al., 2009) (Kovacs et al., 2009)
<i>FUS</i> (fused in sarcoma)	ALS (Amyotrophic lateral sclerosis)	Missense, nonsense, in/del, splicing	Exons + splice sites	RNA gain of function	FUS protein aggregates	(Vance et al., 2009) (Kwiatkowski et al., 2009)
		Missense, nonsense	Exons	RNA loss of function	Unknown	(Merner et al., 2012)
		Missense	Exons, splice sites	Unknown	Unknown	(Vance et al., 2009) (Kwiatkowski et al., 2009)