Published in final edited form as:

Prog Neurobiol. 2012 May ; 97(2): 173–189. doi:10.1016/j.pneurobio.2011.10.006.

Neurodegeneration the RNA way

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Abstract

The expression, processing, transport and activities of both coding and non-coding RNAs play critical roles in normal neuronal function and differentiation. Over the past decade, these same pathways have come under scrutiny as potential contributors to neurodegenerative disease. Here we focus broadly on the roles of RNA and RNA processing in neurodegeneration. We first discuss a set of “RNAopathies”, where non-coding repeat expansions drive pathogenesis through a surprisingly diverse set of mechanisms. We next explore an emerging class of “RNA binding proteinopathies” where redistribution and aggregation of the RNA binding proteins TDP-43 or FUS contribute to a potentially broad range of neurodegenerative disorders. Lastly, we delve into the potential contributions of alterations in both short and long non-coding RNAs to neurodegenerative illness.

Keywords

miRNA; TDP-43; Fragile X; Bidirectional transcription; CAG RNA; Repeat expansion disorders

1. Introduction

As the world population ages over the coming decades, the medical system will face an ever increasing incidence of neurodegenerative disorders. It is estimated that one in 7 people in the US will develop a neurodegenerative disorder in their lifetime, and dementia is now the 6th leading cause of death in the US (Dorsey et al., 2007; Thies and Bleiler, 2011). Neurodegeneration is the broad term used to describe the progressive loss first of neuronal function, then of the neurons themselves. As a central component of many forms of neurodegenerative disease is the presence of protein aggregates, much research to date has focused on protein quality control mechanisms in an effort to understand and possibly treat these disorders (Williams and Paulson, 2008; La Spada and Taylor, 2010; Selkoe, 2011). An emerging avenue of research in neurodegeneration focuses instead on the mechanisms by which RNA and RNA processing contribute to neuronal dysfunction and death. For a subset of diseases, direct mRNA toxicity via a gain of function mechanism has been proposed (Osborne and Thornton, 2006; O'Rourke and Swanson, 2009; Todd and Paulson, 2010). However, for a broader set of disorders, alterations in non-coding RNA, RNA splicing and RNA binding protein activity accompany or drive the neurodegenerative process in novel ways (Gallo et al., 2005; Cooper et al., 2009; Lagier-Tourenne et al., 2010). Moreover, the possibilities for transcriptional control mediated by long non-coding RNAs are just

beginning to come to light, suggesting an even more complex mechanism by which disturbances at the RNA level can contribute to degeneration of the nervous system (Wapinski and Chang, 2011).

This review provides a broad summary of the myriad of ways by which both coding and noncoding RNAs and RNA binding proteins contribute to neurodegenerative disease. Our goal is to provide both a topical introduction for the uninitiated, as well as to facilitate cross-talk among researchers within the various subtopics we cover. We believe that there is likely significant and underappreciated overlap across different neurodegenerative disorders in terms of the mechanisms by which RNA contributes to disease pathogenesis, as evidenced by recent studies in ALS and frontotemporal dementia (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Given the breadth of this topic, aspects of our coverage of specific areas are at times limited or superficial. For each subtopic, we have therefore referenced numerous excellent recent reviews that should allow for more in-depth exploration.

2. Function and processing of RNAs in the central nervous system

To appreciate how alterations in the processing and expression of coding and non-coding RNAs contribute to neurodegeneration, it is important to review recent advances in our understanding of how RNA participates in the regulation of gene expression, RNA processing, and protein translation.

The human transcriptome is made up of both protein coding messenger RNAs (mRNAs) and multiple different classes of non-coding RNAs (ncRNAs), including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear and nucleolar RNAs (snRNAs and snoRNAs, respectively), microRNAs (miRNAs), larger non-coding transcripts (e.g. lincRNAs and lncRNAs), and a host of recently described RNA species whose functions are less clear (e.g. vault RNAs, Y RNAs, and piRNA) (Cooper et al., 2009). Of the human genome, only about 1.2% of the sequence is protein-coding (Lander et al., 2001; Clark et al., 2011). Despite this, a much larger portion (up to 90%) of the genome is transcribed, indicating that a large number ncRNAs likely participate in numerous physiological processes in all cell types, including neurons (Lander et al., 2001; Birney et al., 2007; Wilhelm et al., 2008; Clark et al., 2011). Furthermore, many genes are transcribed in both the sense (i.e. in the direction that yields a protein encoding mRNA) and anti-sense (usually producing a shorter non-coding sequence) directions. The functions of antisense transcription are incompletely understood, but likely involve the regulation of transcription, stability, and translation of the sense transcripts with which they are associated (Katayama et al., 2005).

Messenger RNAs are initially transcribed as a pre-mRNA containing a 5' untranslated region (5' UTR), a 3' untranslated region (3' UTR) and numerous non-coding intronic regions (introns) between the protein coding regions of the mRNA. This pre-mRNA is spliced peri-transcriptionally, such that the intronic sequences are removed and variable combinations of the exons, including the 3' and 5' UTRs, are compiled into a complete coding sequence for translation (Pandit et al., 2008). For each pre-mRNA, there are usually multiple different potential alternatively spliced mature mRNAs produced, with different mature mRNAs being favored in specific tissue and cell types during distinct stages of development. Similarly, exonic combinations can be modulated in response to specific environmental cues (Poulos et al., 2011). The mature mRNA is then transported out of the nucleus and into the cytoplasm as part of a messenger ribonucleoprotein particle (mRNP). There, it associates with ribosomal machinery and is translated into its protein product in a regulated fashion. From initial transcription as a pre-mRNA and through its splicing,

transport, translation, and degradation, the mRNA is associated with numerous RNA binding proteins and ncRNAs that regulate its processing, stability and translation.

Non-coding RNAs and their associated binding proteins are intimately involved in numerous cellular pathways. These pathways, which include RNA processing as well as regulation of transcription and translation, are critical determinants of neuronal differentiation and plasticity. Perhaps not surprisingly, alterations in these pathways have now been identified that contribute to a wide variety of disorders, including a number of neurodegenerative diseases (Cooper et al., 2009). For example, microRNAs (miRNAs) play a critical role in controlling gene expression post-transcriptionally. miRNAs are short sequences of between 20 and 30 nucleotides that are initially transcribed as longer primary miRNAs (pri-miRNAs) containing a hairpin structure, which must be enzymatically cleaved by Drosha, a nuclear RNaseIII (Zeng et al., 2005; Zeng and Cullen, 2006; Kim et al., 2009). The Drosha cleaved sequence, known as a precursor miRNA (pre-miRNA), is then transported to the cytoplasm, where it is cleaved by another RNase, Dicer, to produce the mature miRNA (Ketting et al., 2001; Yi et al., 2003; Murchison and Hannon, 2004; Kosik, 2006; Kim et al., 2009). These mature miRNAs then bind target sequences of mRNA transcripts to mediate either translational repression or mRNA degradation (Kosik, 2006). Interestingly, recent reports suggest that these miRNA-mRNA interactions can also lead to translational activation or transcriptional stimulation under certain circumstances (Kim et al., 2009).

In addition to short ncRNAs such as miRNAs, there are numerous classes of long non-coding RNAs (lncRNAs), including large intergenic non-coding RNAs (lincRNAs) that may meaningfully impact neurodegeneration. LincRNAs are non-protein coding genes which are highly conserved, transcriptionally regulated, spliced, and impart their function as RNA (Guttman et al., 2009). Between 2.3 and 17.2 kb, most lincRNAs studied to date function to modulate the transcription of target protein coding genes or participate in large scale shifts in the epigenetic landscape, as occurs in X-inactivation (Brannan et al., 1990; Brown et al., 1991; Mercer et al., 2009; Ponting et al., 2009; Huarte et al., 2010). Although the specific functions of most lncRNAs in the nervous system are not known, some evidence suggests further roles in neuronal differentiation and regulation of dendritic mRNA translation (Zalfa et al., 2003; Lin et al., 2008; Guttman et al., 2009; Huarte et al., 2010).

Longer variations of non-coding RNAs also include long interspersed nuclear elements (LINEs). LINE1 (or L1 elements) are typically about 6 kb in length, and may comprise up to 20% of the mammalian genome (Scott et al., 1987; Lander et al., 2001; Waterston et al., 2002; Gibbs et al., 2004). L1 elements are transcribed and exported from the nucleus, and contain two open reading frames which encode for a nucleic acid chaperone and a reverse transcriptase; however the majority of the transcribed sequence is not translated (Feng et al., 1996; Martin, 2006). The generation of these proteins allows L1 elements' autonomy for further retrotransposition (Feng et al., 1996; Martin, 2006; Singer et al., 2010). Once in the cytoplasm, L1 elements interact with RNA binding proteins, which includes their translated nucleic acid chaperone and reverse transcriptase which binds *in cis*, and are transported back into the nucleus (usually during mitosis) (Singer et al., 2010). In the nucleus, an endonuclease nicks target DNA sequences and allows reverse transcription of the L1 into the genome (Luan et al., 1993; Cost and Boeke, 1998). This process of L1 duplication and retrotransposition can alter the transcription of neighboring genes, and has recently been shown to play a role in neurodevelopment (Singer et al., 2010).

3. RNA dominant neurological disorders

3.1. RNA as a toxic species: the sequestration hypothesis

The concept that RNA itself acts as a primary toxic species in a neurological disorder was first proposed and established for myotonic dystrophy type 1 (DM1). DM1 is the most common adult onset muscular dystrophy and the third most common overall (Philips et al., 1998; Mankodi et al., 2000; Liquori et al., 2001; Kanadia et al., 2003; Wheeler and Thornton, 2007). In addition to skeletal muscle weakness and myotonia, patients with DM1 also have complications related to cardiac function, cataracts, and neuropsychiatric symptoms including cognitive dysfunction (Griggs and Wood, 1989; Jaspert et al., 1995; Modoni et al., 2004; Wheeler and Thornton, 2007). An autosomal dominant disorder, DM1 results from an expanded CTG repeat in the 3' UTR of the DMPK gene (Brook et al., 1992; Fu et al., 1992; Mahadevan et al., 1992). Initial evaluations tested whether this repetitive sequence impaired production of the DMPK protein which contained the repeat, or if it may alter the expression of the genes surrounding the locus of the mutation. However, the expanded CTG repeats do not significantly alter DMPK protein expression and altered expression of neighboring genes did not recapitulate key features of the clinical disorder (Jansen et al., 1996; Reddy et al., 1996; Klesert et al., 2000; Sarkar et al., 2000; Filippova et al., 2001). However, expression of CUG repeat RNA in isolation was capable of eliciting cytotoxicity and recapitulating many of the central aspects of the human disease (Taneja et al., 1995; Philips et al., 1998; Mankodi et al., 2000). CUG repeat mRNAs form nuclear foci that co-localize with multiple members of the muscle blind-like (MBNL) splicing factor family in animal models and in affected tissues from patients (Miller et al., 2000; Mankodi et al., 2003). MBNL binds to CUG RNA repeats and redistributes from a diffuse pattern of nuclear staining to punctate foci in both patient tissue samples and when co-expressed with CUG RNA in cell culture models (Taneja et al., 1995; Mankodi et al., 2000; Miller et al., 2000). Overexpression of MBNL in mouse or *Drosophila* animal models of DM1 suppresses multiple aspects of the CUG RNA phenotype (Kanadia et al., 2006; Vicente-Crespo et al., 2008). MBNL knockout mice recapitulate many key features of the DM1 phenotype, including myotonia, dystrophic muscle changes, and some shared splicing abnormalities (Kanadia et al., 2003, 2006). Based on these findings, it was proposed that expanded repeats of CUG RNA might act to sequester MBNL and perhaps other key proteins as a primary disease mechanism (Mankodi et al., 2000, 2003; Miller et al., 2000).

There is significant evidence to support the hypothesis that MBNL and other proteins may contribute to DM1 pathology. In DM1 tissues, there is missplicing of a number of MBNL target messages in a fashion consistent with loss of MBNL function (Savkur et al., 2001; Mankodi et al., 2002; Wheeler et al., 2007; Osborne et al., 2009). These include missplicing of the chloride channel *ClC1*, which leads to myotonia; a splice variant of the glutamate receptor *NMDAR1* that is predicted to impair proper dendritic targeting of the mRNA; and multiple splicing alterations in the microtubule-associated protein tau that may influence this protein's stability and aggregation properties (Sergeant et al., 2001; Mankodi et al., 2002; Jiang et al., 2004; Leroy et al., 2006; Wheeler et al., 2007; Dhaenens et al., 2008, 2011; Ghanem et al., 2009; Itoh et al., 2010). The best established of the MBNL dependent missplicing events in DM1 is the retention of a destabilizing splice variant in the chloride channel *ClC1*, which leads to myotonia (Mankodi et al., 2002; Wheeler et al., 2007). However, for most other identified splicing alterations, the evidence that these result from MBNL sequestration is associative rather than direct. This is especially important because of recent evidence indicating splicing alterations in several unrelated muscular dystrophy models, suggesting that muscle regeneration itself may lead to some of the observed alternative splicing changes (Orengo et al., 2011).

Identification of the gene responsible for myotonic dystrophy type 2 (DM2) further supports a critical role for MBNL sequestration in DM1 pathology (Liquori et al., 2001). DM2 has a clinical phenotype that is similar to DM1, although there is no congenital form of the disease and only minimal central nervous system involvement. DM2 results from a CCTG expansion in an intronic region of an unrelated gene, zinc finger nuclease 9 (Liquori et al., 2001). When transcribed, these CCUG repeats act very much like their counterparts in DM1, forming nuclear RNA foci that sequester MBNL proteins and are associated with similar splicing defects (Liquori et al., 2001; Mankodi et al., 2001). Taken together, DM1 and DM2 present the strongest case for a primary sequestration model of toxicity in RNA dominant disorders.

However, MBNL1 sequestration is only part of the DM1 story. In addition to causing splicing deficits, studies in patient derived tissues and multiple animal models of the disease demonstrate alterations in CUG binding protein 1 (CUGBP1) expression and activation (Philips et al., 1998; Timchenko et al., 2001). CUGBP1 is also a splicing factor, but it has numerous other roles in RNA processing, transport, and translation within neurons. Although CUGBP1 can bind to CUG RNA, it is not sequestered in RNA foci and instead appears to be activated by the presence of expanded CUG RNA expression. Importantly, overexpression of CUGBP1 recapitulates some critical features of DM1 pathology (Ward et al., 2010). Mice expressing 8 times the endogenous levels of CUGBP1 show significant skeletal muscle wasting, as evident by decreased muscle weight and functional stamina, histological evidence of degeneration, and alternative splicing (Ward et al., 2010). Together, these data suggest a concurrent role for MBNL and CUGBP1 in DM1 (Wang et al., 2007; Orengo et al., 2008).

In addition, the phenotypic differences between DM1 and DM2, especially in relation to cognitive decline and the congenital phenotype, indicate that further MBNL independent mechanisms are important in DM1. Indeed, comparison of transcriptional profiles from muscle taken from MBNL knockout and expanded CUG RNA transgenic mice reveal significant differences in transcriptional regulation that suggest roles for other factors (Osborne et al., 2009; Du et al., 2010). Some recent lines of evidence suggest roles for the sequestration or aberrant activation of MBNL independent pathways. In cardiac tissue, expression of a transgene containing the 3' UTR of DMPK with an unexpanded repeat recapitulates many of the cardinal features of DM1 (Mahadevan et al., 2006). Both DM1 patients and these mice demonstrate upregulated expression of the cardiac specific transcription factor NKX2-5 in cardiac and non-cardiac tissues, leading to myocyte conduction defects due to aberrant transcription of other genes, including connexins 40 and 43 (Mahadevan et al., 2006; Yadava et al., 2008). These effects appear to be MBNL independent, as they are not associated with RNA foci formation or MBNL inclusions.

RNA binding protein sequestration is not unique to DM1 and DM2. In total, sequestration of specific RNA binding proteins is proposed as a primary pathogenic mechanism in at least five different RNA dominant disorders (see Table 1). One recently described example of this is spinocerebellar ataxia type 10 (SCA10). SCA10 is a rare cerebellar ataxia and epilepsy syndrome that results from an ATTCT repeat expansion in the 3' UTR of the E46L gene (Matsuura et al., 2000; Lin and Ashizawa, 2003; White et al., 2010). In patient derived cells and in transfected cells, this RNA repeat drives formation of nuclear and cytoplasmic foci and cellular apoptosis (White et al., 2010). The RNA foci co-localize with the RNA binding protein hnRNP K and strongly bind to it *in vitro* and *in vivo* (White et al., 2010). Moreover, central aspects of the cellular RNA toxicity can be recapitulated by siRNA knockdown of hnRNP K (White et al., 2010). Future work will need to address whether interactions of AUUCU repeat RNA with hnRNP K are sufficient to explain the full clinical phenotype in

animal models and whether overexpression of hnRNP K can suppress the cellular toxicity elicited by the AUUCU repeat RNA (White et al., 2010).

Another form of spinocerebellar ataxia has recently been described, SCA31 (Sato et al., 2009). SCA31 arises from an inserted TGGAA repeat on chromosome 16. When this repeat is transcribed it forms nuclear inclusions and co-localizes with the serine/ arginine-rich splicing factors (SFRS) 1 and 9 (Sato et al., 2009). While this finding does not yet directly link SFRS1 and 9 inactivity to the pathology associated with SCA31, it does indicate yet another neurological disorder may be associated with nucleotide repeats capable of mediating neurodegeneration by way of protein sequestration.

3.2. Many roads lead to neuronal dysfunction and death in FXTAS

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder which primarily affects the grandfathers of children with Fragile X Syndrome (FXS). FXS, the most common known inherited form of mental retardation, results from a large expansion of a CGG trinucleotide repeat in the 5' UTR of the *FMR1* gene on the X chromosome (Oberle et al., 1991; Verkerk et al., 1991; Yu et al., 1991). CGG expansion to greater than 200 repeats induces hypermethylation of the repeat sequence and a neighboring CpG island within the *FMR1* promoter, preventing transcription of *FMR1* mRNA and leading to the absence of the *FMR1* protein product, FMRP (Bell et al., 1991; Pieretti et al., 1991; Feng et al., 1995). This expansion from a normal repeat size of 6–45 CGGs to a full mutation range repeat takes at least two generations (Fu et al., 1991). While initially thought to be asymptomatic, carriers of a “pre-mutation” range repeat of 55–200 CGGs have a distinct clinical and molecular phenotype (Hagerman et al., 2001). Specifically, FXTAS patients acquire a progressive neurodegenerative disorder characterized by action tremor, gait difficulties, neuropsychiatric symptoms, peripheral neuropathy and dementia after the age of 50 (Hagerman et al., 2001; Basuta et al., 2011).

Pathologically, FXTAS patients display widespread neurodegeneration and brain atrophy with intranuclear ubiquitin positive inclusions in neurons and astrocytes (Greco et al., 2002; Iwahashi et al., 2006). Further, pre-mutation CGG repeat expansions lead paradoxically to increased expression of *FMR1* mRNA and decreased expression of FMRP, depending on the size of the repeat expansion and the tissue type sampled (Tassone et al., 2000a, b; Kenneson et al., 2001). The increase in expression of the CGG repeat mRNA is significant, often as high as 5-fold greater in patient derived cell lines or in animal models of the disease. This increased *FMR1* RNA expression results from greater *FMR1* transcription and not from an increase in *FMR1* mRNA stability associated with the enlarged repeat (Tassone et al., 2000a, b, 2007). Evidence now suggests this augmented transcription is triggered by epigenetic alterations induced by the CGG repeat expansion itself as DNA (Todd et al., 2010). Specifically, CGG repeat expansions elicit chromatin changes *in vitro*, in a *Drosophila* model of the disease, and in patient derived cell lines. Interestingly, these alterations in local chromatin structure are dynamic and modifiable by genetic and pharmacologic means, suggesting that agents aimed at chromatin remodeling might have therapeutic potential in FXTAS (Todd et al., 2010).

FXTAS pathogenesis is hypothesized to result from a primary mRNA toxic gain of function mechanism akin to DM1 (Hagerman et al., 2001; Todd and Paulson, 2010). *FMR1* mRNA but not FMR protein was found in FXTAS patient derived nuclear inclusions (Tassone et al., 2004). Further, ectopic expression of an expanded CGG repeat in the context of a heterologous transcript was sufficient to cause neurodegeneration and intranuclear inclusions in *Drosophila* (Jin et al., 2003), transfected cells (Hashem et al., 2009), and in murine purkinje cells (Hashem et al., 2009). However, unlike DM1, the primary sequestered RNA binding protein in FXTAS remains unclear. A proteomic analysis of the nuclear

inclusions in FXTAS revealed a large number of proteins in the aggregates, including the RNA binding protein hnRNP A2/B1, the nuclear envelope protein lamin A/C and the small heat shock protein α B-crystallin (Iwahashi et al., 2006). Importantly, a number of other potential toxic aggregating proteins were not seen in the inclusions, including α -synuclein and tau, although TDP-43 was not specifically tested (see Section 4). Subsequently, Jin and colleagues identified two CGG repeat RNA binding proteins, Pur α and hnRNP A2/B1, by affinity chromatography (Jin et al., 2007). Both are present in FXTAS patient inclusions and co-expression of either of these proteins suppresses CGG repeat mediated toxicity in a *Drosophila* model (Jin et al., 2007; Sofola et al., 2007a, b). Interestingly, hnRNP A2/B1 binds to CUGBP1 and overexpression of CUGBP1 suppresses the CGG repeat phenotype in *Drosophila* (Sofola et al., 2007a, b). However, as the changes in CUGBP1 are in the *opposite* direction of those seen in DM1, the relevance of this finding to FXTAS pathogenesis remains unclear (Sofola et al., 2007a, b).

More recent evidence suggests that altered splicing is important in FXTAS pathogenesis. Sam68 is another RNA binding protein identified within FXTAS human patient brain inclusions (Sellier et al., 2010). Sam 68 binds to both RNA and DNA and is a known splicing factor (Richard, 2010). In cell culture, Sam 68 is recruited to CGG repeat aggregates as an early event in pathogenesis. This sequestration leads to altered splicing of a number of Sam68 target mRNAs, which are also misspliced in FXTAS model mice and human patient brain samples (Sellier et al., 2010). Moreover, knockdown of Sam68 prevented CGG RNA induced aggregate formation. Interestingly, Sam68 does not bind directly to the CGG RNA, suggesting that an intermediate CGG binding protein is required to facilitate this initial interaction (Sellier et al., 2010).

Although sequestration of Sam68 and other RNA binding proteins by CGG repeat RNA may be an important aspect of FXTAS pathogenesis, other mechanisms likely contribute to neuronal dysfunction and neurodegeneration in this disorder. There is evidence for a failure of protein quality control pathways and altered cellular homeostasis in FXTAS model systems and in patient samples as evidenced by the presence of large proteinaceous ubiquitinated intranuclear inclusions (Greco et al., 2002; Willemsen et al., 2003). In a *Drosophila* model of the disease, co-expression of the chaperone heat shock protein 70 (Hsp70) suppresses CGG repeat induced neurodegeneration (Jin et al., 2003). Similarly, in patient derived fibroblast cultures and in neuronal cultures from female heterozygous FXTAS model mice, there are significant markers of cellular stress, including elevated expression of multiple heat shock proteins, increased nuclear heterochromatin formation and decreased cellular viability (Garcia-Arocena et al., 2010; Chen et al., 2009). In HEK cells stably transfected with 120 CGG repeats, gene activation triggers reduced cell viability and an increased sensitivity to drugs that impair protein quality control pathways (Handa et al., 2005). In transfected human neuroblastomas containing the *FMRI* 5' UTR with 88 repeats in front of eGFP, there was a similar reduction in viability associated with gene activation and α B-crystallin positive (but ubiquitin negative) intranuclear inclusion formation at 72 h after gene transfection (Arocena et al., 2005). Interestingly, in both patient derived fibroblasts as well as transfected cells, CGG repeat expression leads to alterations in the expression and distribution of lamin A/C within the nuclear membrane (Garcia-Arocena et al., 2010; Arocena et al., 2005). Given that lamin A/C is found as a component of the intranuclear inclusions, the authors proposed that one component of FXTAS pathogenesis could be as a "laminopathy", as occurs in Emory-Dreifuss muscular dystrophy and the premature aging disorder Progeria (Arocena et al., 2005; Capell and Collins, 2006; Garcia-Arocena et al., 2010; Zaremba-Czogalla et al., 2011).

Although FXTAS is a degenerative disease that affects primarily a geriatric population, a number of recent studies suggest that the CGG pre-mutation also alters neuronal and

synaptic development. To understand the relevance of these findings, it is important to appreciate the normal functions of FMRP, the dynamics of FMR1 translation under normal circumstances and the potential effect of the expanded CGG repeat on translational inefficiency. FMRP itself is an RNA binding protein that regulates dendritic synthesis of proteins in response to synaptic stimulation (Ronesi and Huber, 2008). Interestingly, one of the messages FMRP likely regulates at synapses is FMR1, which is rapidly translated in response to metabotropic glutamate receptor stimulation (Weiler et al., 1997; Todd et al., 2003; Hou et al., 2006). Thus, efficient translation of FMRP at synapses is an important part of the protein's basal function. The CGG repeat in FMR1 acts to impair translation by interfering with ribosomal scanning through the 5' UTR, preventing appropriate loading of CGG expanded FMR1 mRNA into polyribosomal complexes (Primerano et al., 2002; Ludwig et al., 2009, 2011). Although FMRP expression levels in patients with FXTAS have been widely reported as only slightly decreased, most of these measurements have been done on lymphocytes or whole blood samples rather than on brain tissue, where changes might be expected to be most robust (Tassone et al., 2000a, b; Kenneson et al., 2001; Primerano et al., 2002; Singh et al., 2007). Given evidence from mice that FMRP expression declines with age (Singh et al., 2007), a role for FMRP insufficiency in FXTAS, especially in patients with large CGG repeat expansions, deserves further consideration (Hessl et al., 2011).

Most of the studies addressing a potential role for FMRP insufficiency have utilized one of two knock-in (KI) mouse models where a portion of the human 5' UTR from a pre-mutation carrier has been inserted into the *FMR1* mouse locus (Bontekoe et al., 2001; Peier and Nelson, 2002; Entezam et al., 2007; Hashem et al., 2009). Both mice, as well as a YAC transgenic model mouse, demonstrate intra-generational instability, elevated FMR1 mRNA expression and variably decreased FMRP expression at larger premutation repeat lengths (Peier and Nelson, 2002; Willemsen et al., 2003; Entezam et al., 2007; Brouwer et al., 2008; Hashem et al., 2009).

In the best characterized of the models (Willemsen et al., 2003; Berman and Willemsen, 2009), CGG KI mice show signs of hippocampal-dependent cognitive impairment at an early age that precedes frank neurodegeneration or inclusion formation (Hunsaker et al., 2009). These same mice also show signs of age dependent cognitive decline in later life as measured by impaired visual-spatial learning and performance on the Morris water maze (Van Dam et al., 2005). Interestingly, cultured hippocampal neurons from heterozygous female CGG KI mice demonstrate early developmental defects including delayed dendritic complexity and outgrowth (Chen et al., 2010). As a follow-up to this study, Cunningham and colleagues identified significant delays in cortical development in CGG KI mice during embryogenesis characterized by migrational defects in the neocortex and altered expression of neuronal lineage markers (Cunningham et al., 2010). As these mice have relatively large CGG repeat expansions (~150 CGGs), they express only about 50% of basal FMRP levels in embryonic tissues. Importantly, defects in neural stem cell proliferation, which have been reported in *Fmr1* knockout mouse models (Tervonen et al., 2009), were not present in these animals, suggesting the etiology of the developmental defects in the CGG KI mice may be more than just FMRP insufficiency (Cunningham et al., 2010).

In the more recently described of the two knock-in models, the inefficiency of CGG-Fmr1 translation for a given repeat size appears to be greater (Entezam et al., 2007). This translational inefficiency leads to lower FMRP levels for a given CGG repeat size, especially in certain brain regions, though greater basal CGG Fmr1 RNA expression (Entezam et al., 2007). Despite the increased amount of CGG-containing RNA produced, these mice generate few intranuclear inclusions, although they do demonstrate mild cerebellar and cortical neurodegeneration with age (Entezam et al., 2007). In a recent study,

these mice were found to exhibit some features also seen in *Fmr1* KO mice. Most notably, they had decreased dendritic arborization and longer thin spines on histological analysis, along with increased regional brain protein synthesis rates (Qin et al., 2011). Taken together, these data suggest that therapeutic strategies aimed selectively at eliminating the toxic CGG repeat mRNA run a significant risk of exacerbating symptoms of FXTAS that may relate to decreased basal or activity dependent FMRP expression (Todd et al., 2010).

3.3. Bidirectional transcription of nucleotide repeats

Spinocerebellar ataxia type 8 (SCA8) is an autosomal dominant disorder characterized by cerebellar ataxia and progressive cerebellar neurodegeneration. Early work on SCA8 suggested yet another example of an RNA binding protein sequestration mechanism (Koob et al., 1999). SCA8 arises from a CTG expansion in the 3' end of a non-protein coding RNA, Ataxin 8OS. As in DM1, this CUG RNA expansion leads to nuclear RNA foci formation and MBNL sequestration in the cerebellum, with associated splicing changes in the GABA-A transporter 4 (GAT4) in patient tissue samples and animal models (Daughters et al., 2009). Additionally, bidirectional transcription at the SCA8 locus through the expanded repeat mutation has been observed (Moseley et al., 2006). The antisense transcript, Ataxin8, includes a short cryptic open reading frame that includes the CAG repeat in the glutamine reading frame (Moseley et al., 2006). Furthermore, the identification of poly-glutamine inclusions in both animal models and human brain tissues suggests that SCA8 may also be a polyglutamine disorder, akin to numerous other spinocerebellar ataxias (Moseley et al., 2006).

Huntington disease-like 2 (HDL2) provides another example of a toxic effect possibly due to antisense gene transcription. HDL2 is a neurodegenerative disorder that is clinically and pathologically similar to Huntington's disease, including the presence of 1C2 positive (presumably polyglutamine) aggregates (Rudnicki et al., 2008). However, HDL2 results from a CTG expansion in the 3' UTR of the Junctophilin gene. As in SCA8, initial research supported a mechanism related to RNA foci, MBNL recruitment and splicing defects (Rudnicki et al., 2007, 2008). In recently published work using a BAC transgenic mouse model of the disease, Wilburn and colleagues established that there is an antisense CAG transcript generated which is translated to produce a polyglutamine peptide (Wilburn et al., 2011). They further demonstrated that even when the CUG sense transcript is silenced, the CAG antisense transcript is still generated and is sufficient to drive pathogenicity and the behavioral phenotypes observed. These findings suggest a concurrent mechanism in which a toxic gain-of-function RNA and a deleterious polyglutamine protein can be generated from the same repeat expansion, but that, in the case of HDL2, protein derived toxicity appears to be the most relevant (see Fig. 1).

Bidirectional transcription at the *FMR1* locus may also play a role in pathology of FXTAS. One antisense transcript has been discovered in human lymphoblastoma cells which overlaps *FMR1* in exon 1, and is transcribed through the CGG repeats in the 5' UTR (Ladd et al., 2007). This antisense transcript, deemed ASFMR1, is spliced, processed, and exported from the nucleus (Ladd et al., 2007). ASFMR1 contains an open reading frame that, if utilized, would encode for a polyproline tract through the CGG/CCG repeat. In addition to evidence of an alternative splice site in FXTAS patient ASFMR1, these data suggest an alternate role for aberrant protein generation in FXTAS pathology (Ladd et al., 2007). However, ASFMR1 is not the only antisense transcript of *FMR1*; an additional transcript, FMR4, has been described that overlaps only the very 5' end of the *FMR1* gene (Khalil et al., 2008). FMR4 does not regulate *FMR1* expression, though as with ASFMR1, its expression is enhanced in FXTAS patient tissues (Khalil et al., 2008). Interestingly, FMR4 ncRNA functions in cell cycle regulation and over expression of FMR4 lead to increased cell proliferation *in vitro* (Khalil et al., 2008). These data suggest yet another potential

component contributing the pathology of FXTAS, especially in light of transcriptional upregulation at the *FMR1* locus.

In addition to these three disorders, there is evidence of bidirectional transcription through the nucleotide repeat sequence in other nucleotide repeat diseases, including DM1 and spinocerebellar ataxia type 7 (Cho et al., 2005; Sopher et al., 2011). These antisense RNAs clearly influence transcription of the sense transcript and perhaps the epigenetic landscape of the associated genes, but they may also have important potential roles in toxicity. Future studies will be needed to define how each of these transcripts influences disease pathogenesis.

3.4. CAG repeats and RAN translation: novel disease mechanisms for nucleotide repeat disorders

The potential toxicity of CAG RNA repeats is relevant to a number of neurodegenerative diseases (some of which are discussed above), and as such a number of recent studies focused on CAG toxicity both within disease context and in isolate. Early work demonstrated the ability of proteins to bind to cytoplasmic CAG RNA repeats in a length-dependent fashion (McLaughlin et al., 1996). In more recent work, MBNL is capable of binding to ectopically expressed CAG repeat RNA in *Caenorhabditis elegans* (L.C. Wang et al., 2011). Similarly, a *Drosophila* model of spinocerebellar ataxia 3 expressing a non-coding expanded CAG repeat elicited neurodegeneration (Li et al., 2008). Interestingly, up-regulating the fly MBNL homolog caused a marked increase in polyglutamine-containing ataxin3 neurodegeneration, which is the opposite direction of interaction to that seen with the CUG repeat expression (Houseley et al., 2005; Li et al., 2008; Braida et al., 2010; Wojciechowska and Krzyzosiak, 2011). This effect was also investigated in a mouse model which expressed CAG repeats in the 3' UTR of a green fluorescence protein reporter (Hsu et al., 2011). In this model, longer CAG repeats formed RNA foci and led to functional abnormalities in skeletal muscle and sperm which expressed the transgene (Hsu et al., 2011).

CAG repeats form hairpin structures and RNA foci with MBNL in human Huntington patient fibroblasts (de Mezer et al., 2011). Interestingly, *in vitro* evidence also suggests that CAG repeat RNA hairpins can be cleaved by Dicer and made into functional miRNAs which could have broad-scale effects on other target gene regulation (Krol et al., 2007). Similarly, expression of bidirectional transcripts may also contribute to toxicity via this mechanism. In *Drosophila*, expressing both CAG and complementary CUG expanded repeats results in increased toxicity as a result of dysregulated transcription of numerous genes (Krol et al., 2007). These complementary CTG expanded repeats result in the generation of miRNA-like sequences (Yu et al., 2011). Surprisingly, this increased toxicity with co-expression of CUG and CAG transcripts is the exact opposite of effects seen when CGG and CCGRN As are co-expressed in *Drosophila*, where dsRNA duplex formation triggers transcript degradation through an Argonaute dependent pathway, leading to phenotypic rescue (Sofola et al., 2007a, b). Thus, it appears that CAG RNA repeats are capable of eliciting toxicity in a variety of systems, either in isolation, or as a duplex with CUG RNA. However, given the overwhelming evidence for toxicity of polyglutamine containing proteins in neurons (Williams and Paulson, 2008), it remains unclear whether CAG RNA toxicity in these disorders will emerge as a significant or minor contributor to pathogenesis.

Even more provocative is a recent study suggesting a novel mechanism by which non-coding RNA repeats might lead to toxicity and neurodegeneration. Zu et al. describe an aberrant repeat-associated non-ATG (RAN) translation which generates polyglutamine, polyalanine, and polyserine tracts from otherwise non-coding CAG repeat mRNA messages (Zu et al., 2011). This was initially identified when transfection of a CAG repeat-containing

Ataxin8OS construct lacking the ATG start site failed to prevent translation of the polyglutamine tract. Further experiments using C-terminal epitope tags in differing reading frames demonstrated that this aberrant translation occurred in all three possible reading frames of the CAG repeat. The effect of RAN translation was repeat length-dependent, in that constructs with 15 CAG repeats did not trigger AUG independent translation, but those with 45 or greater did. This RAN translation only occurs when the repeat tract is complementary and forms a hairpin structure. Zu et al. further identified staining for these novel proteins in a SCA8 mouse model, and in human SCA8 cerebellum using an antibody generated against the putative SCA8-GCA frame (alanine) peptide. A similar approach demonstrated a novel polyglutamine protein in DM1, presumably as a result of RAN translation from an antisense transcript though the CTG/CAG repeat (Cho et al., 2005; Zu et al., 2011). These findings suggest a novel mechanism for aberrant protein synthesis from repetitive nucleotide sequences in the absence of an obvious open reading frame. While further studies are required to establish whether RAN translation significantly contributes to pathogenesis in DM1 and SCA8, the potential extension of this mechanism to other nucleotide repeat disorders could dramatically change our understanding of what drives neurodegeneration in this class of diseases (Pearson, 2011).

4. TDP-43 and FUS as “RNA binding-proteinopathies”

A major area of advancement in neurodegenerative disease research over the past five years involves the identification of two proteins commonly found in neuronal inclusions: the Tar DNA binding protein of 43 kD (TDP-43) and the Fused in Sarcoma/ Translocated in Liposarcoma protein (FUS/TLS) (Lagier-Tourenne and Cleveland, 2009; Buratti and Baralle, 2010; Chen-Plotkin et al., 2010; Gendron et al., 2010; Lagier-Tourenne et al., 2010; Mackenzie et al., 2010). TDP-43 was identified as a component of ubiquitin positive but tau negative inclusions in patients with frontotemporal lobar dementia (FTLD) and in neuronal inclusions in the vast majority of sporadic cases of amyotrophic lateral sclerosis (ALS) (Neumann et al., 2006). Subsequent analysis of familial and sporadic cases of ALS revealed mutations in TDP-43 as a rare cause of the disease (Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Yokoseki et al., 2008). TDP-43 pathology has now been identified in numerous neurodegenerative conditions associated with proteinaceous neuronal and non-neuronal inclusion formation, including Alzheimer's disease, Parkinson's disease, polyglutamine disorders such as Huntington's disease, and Inclusion Body Myopathy (Amador-Ortiz et al., 2007; Guinto et al., 2007; Higashi et al., 2007; Nakashima-Yasuda et al., 2007; Neumann et al., 2007; Hu et al., 2008; Lin and Dickson, 2008; Schwab et al., 2008; Uryu et al., 2008; Weihl et al., 2008; Arai et al., 2009; Finch et al., 2009; Chanson et al., 2010; Lagier-Tourenne et al., 2010; Haraguchi et al., 2011; Pao et al., 2011).

TDP-43 is a ubiquitously expressed 414 amino acid protein with two N-terminal RNA recognition motifs and a flexible glycine rich region in the C terminus that mediates interactions with other proteins (Kuo et al., 2009; Lagier-Tourenne and Cleveland, 2009). Under normal circumstances, TDP-43 is predominantly localized in the nucleus associated with a large number of proteins and mRNAs in heterogeneous nuclear ribonucleoprotein particles (hnRNPs) (Buratti et al., 2005; Freibaum et al., 2010). As a component of these hnRNPs, it binds to both RNA and DNA *in vitro* and *in vivo* (Buratti and Baralle, 2001; Casafont et al., 2009; Lalmanasingh et al., 2011). The exact critical roles of TDP-43 within these complexes are still being elucidated, but evidence to date supports functional roles at multiple steps in RNA expression and processing, including transcriptional regulation, mRNA splicing, RNA transport/stabilization and translation (Ou et al., 1995; Buratti et al., 2001, 2004, 2005; I.F. Wang et al., 2008; Freibaum et al., 2010; McDonald et al., 2011).

In 2009, a second RNA and DNA binding protein, FUS, was identified as a cause of familial ALS (Kwiatkowski et al., 2009; Vance et al., 2009). As with TDP-43, subsequent reports identified it as a component of inclusions in other neurodegenerative disorders, including some forms of frontotemporal dementia (FTD), Basophilic inclusion body disease, and neuronal intermediate filament inclusion disease (Munoz et al., 2009; Neumann et al., 2009; Doi et al., 2010). As with TDP-43, FUS was also found in association with the neuronal intranuclear inclusions seen in a number of polyglutamine disorders (Doi et al., 2010). Importantly, FUS is not a component of inclusions in sporadic ALS and does not co-localize with TDP-43 inclusions in most other disorders (Kwiatkowski et al., 2009; Vance et al., 2009; Lagier-Tourenne et al., 2010). However, there are significant functional similarities between FUS and TDP-43 that have led investigators to consider them as a new class of proteins involved in neuronal inclusion formation rather than solely as distinct entities (Lagier-Tourenne et al., 2010). As with TDP-43, FUS binds avidly to RNA and DNA and is found largely in nuclear RNP complexes, albeit with different protein and RNA partners and a slightly different localization pattern (Zinszner et al., 1997; Hicks et al., 2000). Similarly, FUS is thought to participate in a number of stages RNA transcription and processing, including RNA splicing, transport and stability (Zinszner et al., 1997; Hicks et al., 2000; Gal et al., 2010; Ito et al., 2011).

One of the pathological hallmarks of the ALS and FTL associated with TDP-43 inclusions is the redistribution of TDP-43 from the nucleus and into the cytoplasm (Neumann et al., 2006). This appears to be an early event in the disease process, and can occur in the absence of frank intranuclear or cytoplasmic inclusions in both patients and mouse models (Giordana et al., 2010; Wils et al., 2010). This cytoplasmic redistribution is associated with a significant decrease in nuclear TDP-43 staining (Kwiatkowski et al., 2009; Vance et al., 2009). Interestingly, the redistribution of endogenous TDP-43 can be triggered by expression of mutated forms of TDP-43 lacking a nuclear localization signal (NLS), leading to recapitulation of central features of disease pathology (Winton et al., 2008). Importantly, FUS also redistributes to the cytoplasm in ALS cases resultant from FUS mutations as well as in other degenerative disorders where it aggregates (Kwiatkowski et al., 2009; Vance et al., 2009).

A number of lines of evidence suggest that the cytoplasmic redistribution of TDP-43 or FUS is a fundamental component of disease pathogenesis. In neuronal cultures, the cytoplasmic redistribution of fluorescently labeled wildtype or mutant TDP-43 was predictive of neuronal death (Barmada et al., 2010). Interestingly, death was independent of nuclear or cytoplasmic inclusion formation. In *Drosophila* models, human TDP-43 expression is toxic when expressed in any of a number of model systems, with generally greater toxicity when pathogenic mutants of TDP-43 are expressed (Hanson et al., 2010; Li et al., 2010; Ritson et al., 2010; Estes et al., 2011). This toxicity is enhanced by limiting expression to the cytoplasm and largely alleviated by limiting expression of the protein to the nucleus by eliminating the nuclear localization or exportation sequence, respectively. Recent work using a mouse model expressing human TDP-43 with a mutated NLS also showed greater toxicity compared to expression of wildtype human TDP-43 alone, although this analysis was complicated by dosage issues related to increased hTDP-43 in the NLS mutants compared to wildtype TDP-43 controls (Igaz et al., 2011). Interestingly, these NLS mutants were associated with relatively few inclusions and elicited at least a portion of their toxicity by triggering transcriptional down regulation of the endogenous TDP-43 gene, effectively creating a nuclear TDP-43 null mouse in affected neurons. Neurons expressing the human TDP-43 NLS mutants thus exhibit significant transcriptional abnormalities that mirror those reported in association with small interfering RNA (siRNA) knockdown of TDP-43 (Igaz et al., 2011).

Like TDP-43, overexpression of human FUS is toxic in multiple model systems and is associated with cytoplasmic redistribution and aggregate formation (Chen et al., 2011; Igaz et al., 2011; Ju et al., 2011; Kryndushkin et al., 2011; Lanson et al., 2011; Sun et al., 2011). This toxicity is increased with pathogenic mutations in some but not all model systems, and pathogenic mutations do not appear to alter its intrinsic aggregation properties (Fushimi et al., 2011; Igaz et al., 2011; Lanson et al., 2011; Sun et al., 2011). As with TDP-43, cytoplasmic distribution of FUS is associated with greater toxicity, and blocking cytoplasmic redistribution of the protein effectively abrogates toxicity (Lanson et al., 2011; Sun et al., 2011).

In addition to changes in subcellular localization, the ability of TDP-43 and FUS to bind to RNA appears critical to both their normal functions as well as their ability to induce neurodegeneration. In *C. elegans*, a mutation in either of the two major RNA binding domains in TDP-43 completely blocks its neurotoxic effects (Ash et al., 2010). Similarly, in a yeast model of FUS related toxicity, mutations in the RNA binding protein block toxicity and aggregation (Sun et al., 2011). Moreover, genetic suppressors of FUS toxicity included numerous RNA binding proteins normally found in mRNPs and RNA stress granules (Ju et al., 2011; Sun et al., 2011), further supporting a role for FUS interactions with RNA in pathogenicity.

What do TDP-43 and FUS do in the cytoplasm? Under physiological conditions, both proteins are found in association with RNA granules that transport mRNAs into dendrites (Elvira et al., 2006). For FUS in particular, there is evidence that these proteins play an active role in activity dependent transport and translational regulation at synapses (Belly et al., 2005; Fujii et al., 2005). Consistent with this finding, in mice lacking FUS, there are significant dendritic spine abnormalities, with a decrease in total spine number and an increase in long thin filopodia-like spines (Fujii et al., 2005). Abnormalities in spine development are also seen with decreased TDP-43 expression in *Drosophila* neurons, where TDP-43 has been shown to play an active role in the regulated translation of Futsch/MAP1B mRNA (Godena et al., 2011).

There is also an emerging role for both TDP-43 and FUS in stress granule formation and stability (Colombrita et al., 2009; Bosco et al., 2010). Stress granules are microscopically visible cytoplasmic domains that house RNA binding proteins and their cognate translationally arrested mRNAs (Thomas et al., 2011). Their formation is triggered by a host of cellular insults (oxidative stress, heat shock, toxins, axotomy) and is associated with a rapid redistribution of certain hnRNP proteins from the nucleus into the cytoplasm. Normally their formation is transient, allowing for redistribution of mRNAs for storage, decay or translation after the insult. In response to a variety of cellular insults, TDP-43 and FUS both translocate from the nucleus into stress granules (Colombrita et al., 2009; Bosco et al., 2010; Freibaum et al., 2010; Liu-Yesucevitz et al., 2010; Dewey et al., 2011). Neither is required for stress granule formation, but TDP-43 regulates stress granule dynamics, both through direct recruitment of T-cell intracellular antigen 1 (TIA-1) to stress granules and through regulating the expression of another key stress granule protein, GTPase-activating protein binding protein 1 (G3BP) (McDonald et al., 2011).

Given the significant functional overlaps between TDP-43 and FUS, it is perhaps not surprising that their direct or indirect interaction might be important in disease pathogenicity (Ling et al., 2010; Kryndushkin et al., 2011; Lanson et al., 2011). In cells stably transfected with normal human or mutant TDP-43, FUS was identified by SILAC coupled Mass Spectroscopy and immunoprecipitation studies as a TDP-43 interacting protein (Ling et al., 2010). Interestingly, this interaction was enhanced for TDP-43 mutants (Ling et al., 2010). In yeast, FUS and TDP-43 have a high propensity of co-aggregation that is not seen with

other aggregate-prone proteins (Kryndushkin et al., 2011). In *Drosophila*, FUS expression drives a neurodegenerative eye phenotype that is enhanced by co-expression of normal human or mutant TDP-43 (Lanson et al., 2011). Moreover, FUS and TDP-43 form a functional complex at endogenous expression levels in mammalian cells (Kim et al., 2010). Knockdown of either TDP-43 or FUS leads to decreased expression of histone deacetylase 6 (HDAC6) mRNA and protein (Fiesel et al., 2010; Kim et al., 2010). In the case of TDP-43, maintenance of HDAC6 mRNA expression requires an intact RNA binding domain which is partially lost in mutant forms of TDP-43 (Fiesel et al., 2010). Thus, there is a direct convergence of FUS and TDP-43 in common biochemical pathways, suggesting specific activities of these proteins that may be important in disease pathogenesis.

Given the multiplicity of important cellular processes in which TDP-43 and FUS are involved, it has been somewhat difficult to identify a specific pathway that is critical for ALS disease pathogenesis. An important step towards this end was recently achieved via identification of TDP-43 RNA targets and altered transcriptional and splicing patterns in disease and in the absence of TDP-43 (Polymenidou et al., 2011; Sephton et al., 2011; Tollervey et al., 2011). TDP-43 interacts with a remarkably large fraction of the mouse transcriptome (upwards of 30%), predominately through interactions with UG rich sequences in long intronic regions as well as the 3' UTR regions of mRNAs, especially in the cytoplasm. TDP-43 also associates with a large number of non-coding RNAs and these showed the greatest expression alterations in diseased brain tissue samples, although the overall differences in bound messages were relatively small (Tollervey et al., 2011). These studies also identified approximately 900 misspliced genes in the setting of TDP-43 knockdown and another set of genes whose expression were decreased in this same setting. Although a number of interesting candidate genes emerged from these studies, they provided perhaps more questions than answers at this stage. Importantly, similar experiments to identify RNA targets of FUS are ongoing. Once these are available, identifying areas of significant overlap between the FUS and TDP-43 in terms of transcriptome alterations, splicing changes and RNA targets may allow for a more precise definition of the critical pathogenic cascades shared by these two proteinopathies.

Overall, the findings to date support a model whereby redistribution of TDP-43 and/or FUS from the nucleus to the cytoplasm, either into stress granules or inclusions, leads to both a nuclear loss of function as well as a gain of function phenotype associated with excess cytoplasmic accumulation, including the maintenance and propagation of stress granules or altered cytoplasmic translational regulation (see Fig. 2). The initial trigger for this redistribution remains unclear, but it is tempting to speculate that some chronic cellular insult could trigger ongoing activation of the stress granule response which would favor TDP-43 redistribution. With the identification of specific TDP-43 RNA targets and effects that can be defined as predominantly nuclear or cytoplasmic, it is now becoming possible to more effectively test this hypothesis and identify which, if either, of these two components is critical to disease pathogenesis in TDP-43 associated disorders.

5. MicroRNAs in neurodegeneration

MicroRNAs (miRNAs) are short non-coding RNAs which regulate mRNA stability and translation (Eacker et al., 2009). In addition to direct RNA toxicity and the primary roles of TDP-43 and FUS in neurodegenerative disorders, numerous lines of research now implicate miRNAs and altered miRNA processing in neuro-degeneration (Hebert and De Strooper, 2009; Delay and Hebert, 2011; Enciu et al., 2011). Generally, these studies have taken one of two approaches: studying the impact of global miRNA synthesis on neuronal function and neurodegeneration, or identifying alterations in the expression of particular miRNAs in models of neurodegenerative diseases. However, a few recent studies suggest a model by

which mutated disease proteins interfere with complicated transcription factor/miRNA regulatory networks as a central component of their pathogenic cascades (Packer et al., 2008; Gehrke et al., 2010; Schonrock et al., 2010; Wang et al., 2010).

A number of groups have looked at the effects of suppressing global miRNA processing by knocking down or eliminating critical components of the miRNA machinery, such as Dicer (Bilen et al., 2006; Schaefer et al., 2007; Davis et al., 2008; Lee et al., 2008; Konopka et al., 2010; Tao et al., 2011). Based on these studies, it appears that miRNAs are required for normal neuronal development and function, and their loss eventually leads to neuronal degeneration (Davis et al., 2008; Konopka et al., 2010). In cerebellar purkinje neurons, selective knockout of Dicer postnatally leads to a slowly progressive selective degeneration of these cells starting about 8 weeks post-gene inactivation (Schaefer et al., 2007). Interestingly, these dying neurons recapitulate many common features associated with neurodegenerative disorders, including cytoplasmic inclusion formation, the appearance of autophagic vacuoles, and an increase in apoptotic markers. When Dicer is knocked out in forebrain neurons during development, these animals demonstrate abnormalities in the maintenance of dendrite branching and spine morphology in the hippocampus (Davis et al., 2008). Intriguingly, these deleterious effects of impairing the miRNA machinery are time-dependent (Konopka et al., 2010). If Dicer is knocked out postnatally in these same forebrain neurons, during the first 8 weeks post inactivation, these animals actually display enhanced learning and memory and enhanced hippocampal long term potentiation (Konopka et al., 2010). However, as time progresses, neurodegeneration ensues, suggesting a dichotomous function for miRNA translational control throughout the life of neurons (Schaefer et al., 2007; Konopka et al., 2010). Dicer expression is also critical for the normal function of astroglial populations. As in forebrain neuron knockout experiments, mice lacking Dicer expression in astrocytes develop normally; but display early neurodegeneration consisting of motor abnormalities, seizures, and death by 10 weeks of age (Tao et al., 2011). This finding indicates that miRNA astrocytic function is necessary for neuronal survival (Tao et al., 2011). Broad miRNA alterations also exacerbate neurodegenerative phenotypes in disease models. For example, reducing Dicer function in *Drosophila* increases polyglutamine-mediated cell toxicity (Bilen et al., 2006). Additionally, down-regulation of specific miRNAs which normally target the ataxin1 transcript leads to increased cell death in human cell models of spinocerebellar ataxia type 1, suggesting a normal role for miRNAs in translational regulation of potentially toxic proteins (Lee et al., 2008).

Screening for differences in miRNA expression using either microarray platforms or next generation sequencing technology has facilitated numerous studies into alterations of miRNA levels in neurodegenerative diseases. Altered expression of several miRNAs has been found in both mouse models and human Huntington's disease (HD) patients (Marti et al., 2010; Lee et al., 2011). Among these altered in HD is mir9, which suppresses translation of the RE1-silencing transcription factor (REST) (Packer et al., 2008). REST is normally sequestered in the cytoplasm through its interaction with the huntingtin protein (Zuccato et al., 2003). Polyglutamine expansion in the huntingtin protein abrogates the interaction between huntingtin and REST, allowing REST to translocate to the nucleus and inhibit the transcription of a large group of genes normally suppressed in mature neurons (Zuccato et al., 2007). miR9 itself is downstream of an RE1 repressor sequence, leading to lower miR9 expression in HD patient tissues. This dysregulated feedback loop results in further increased expression of REST, which triggers wide-scale effects on gene transcription in HD (Packer et al., 2008).

A second potential alteration in miRNA pathways in HD involves Drosha, the RNase that cleaves nuclear pri-miRNAs into pre-miRNAs prior to nuclear export. Drosha expression is

decreased in HD patient brains (Lee et al., 2011). Further, there are alterations in the final 3' end sequences in a number of miRNAs in HD, which might alter their stability and regulatory capabilities (Marti et al., 2010). Thus, miRNA-mediated translational regulation in HD could be altered by changes in miRNA processing, rather than just miRNA expression (Marti et al., 2010). However, the mechanism by which this occurs is not currently known.

A number of studies have identified miRNA abnormalities in human samples and animal and cellular models of Alzheimer's disease (AD) (Hebert et al., 2008; Lukiw et al., 2008; Niwa et al., 2008; Patel et al., 2008; W.X. Wang et al., 2008; Boissonneault et al., 2009; Hebert and De Strooper, 2009; Hebert et al., 2009; Lau and de Strooper, 2010; Schonrock et al., 2010; Wang et al., 2010; Yao et al., 2010). Interestingly, direct application of A β 42, the presumed toxic peptide in AD, to neuronal cultures triggers alterations in the expression of numerous miRNAs (Schonrock et al., 2010; Wang et al., 2010). It is not clear how A β 42 elicits this effect, although it is known that A β 42 application activates a number of neuronal receptors and leads to altered synaptic physiology (Freir et al., 2001; Lee et al., 2009; Min et al., 2011). Some evidence also suggests that miRNA alterations in AD can lead to abnormal expression of certain cytoskeletal proteins. Specifically, one study found the actin-binding protein cofilin to be up-regulated in AD, possibly due to reduced levels of miR107 and miR103 (Yao et al., 2010). However, it remains unclear whether miRNA dysregulation is a proximal event in AD pathogenesis or is a downstream consequence of ongoing neurodegeneration that is not specific to AD. One approach to this question is analysis of how miRNA pathways may directly influence A β 42 production. Along these lines, *in vitro* experiments demonstrate a number of specific miRNAs that target APP translation, though these interactions have not yet been demonstrated in human disease tissue (Patel et al., 2008; Hebert and De Strooper, 2009).

A number of recent studies implicate microRNA pathways in the pathogenesis of Parkinson's disease (PD) (Kim et al., 2007; Asikainen et al., 2010; Gehrke et al., 2010; Le et al., 2011). miRNAs are broadly required for dopaminergic neuronal function and survival, as the absence of a critical component of the miRNA machinery, Dicer, leads to apoptosis of dopamine neurons in mice (Kim et al., 2007). Mutations in the leucine rich kinase protein LRRK2 are the most common cause of familial Parkinson's disease (Paisan-Ruiz et al., 2004; Zimprich et al., 2004). A recent study finds that LRRK2 directly effects miRNA processing by binding to Argonaute, a component of the RISC complex. This interaction alters global neuronal miRNA activity and influences neurodegeneration in a *Drosophila* model of PD (Gehrke et al., 2010). A second study implicates miR133, which is reduced in human PD patients, as a particularly important miRNA in Parkinson's disease (Kim et al., 2007). One target for miR133 is the transcription factor Pitx3, which is required for the differentiation and longevity of dopaminergic neurons. Intriguingly, single nucleotide polymorphisms in and near Pitx3 were found to be associated with Parkinson's disease in genome wide association studies (Kim et al., 2007; Le et al., 2011). Pitx3 itself regulates transcription of miR133, creating a feedback loop for Pitx3 expression that may prove pivotal to dopaminergic neurodegeneration in PD (Kim et al., 2007).

6. The new RNAs on the block: a future role in neurodegeneration?

While miRNAs have been widely studied to date, a number of other important classes of non-coding RNAs have not yet been extensively examined for roles in neurodegeneration. Long non-coding RNAs likely play critical roles in neuronal function and thus might be expected to contribute to neurodegenerative processes (Mercer et al., 2008; Qureshi et al., 2010). Long intergenic non-coding RNAs (lincRNAs) are evolutionarily conserved sequences that are transcribed, spliced and polyadenylated in a fashion resembling mRNAs

(Mercer et al., 2009; Ponting et al., 2009; Guttman et al., 2010). lincRNAs are purported to mediate chromatin modifications and transcriptional regulation, although the full spectrum of their potential functions remains unknown (Khalil et al., 2009; Huarte et al., 2010; Tsai et al., 2010; Gong and Maquat, 2011; X. Wang et al., 2011). Specific lincRNAs such as the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and nuclear enriched abundant transcript 1 (NEAT1) are critical not only for correct splicing of other transcripts, but also for proper synapse formation (Bernard et al., 2010; Tripathi et al., 2010). Intriguingly, these two lincRNAs are specifically altered in the setting of TDP-43 pathology in FTD, suggesting an active role in neurodegenerative processes (Tollervey et al., 2011).

To date, there are few other published examples of dysregulated lincRNAs in neurodegenerative disease. One recent study identified the non-coding RNA 17A, which participates in alternative splicing of target mRNAs, as up-regulated in AD patient tissues (Massone et al., 2011). Interestingly, 17A expression leads to an increased secretion of toxic A β peptide, and is capable of facilitating plaque-forming protein aggregates (Massone et al., 2011). Further, in a recent study identifying a novel mechanism of RNA control in AD, Faghihi and colleagues describe a non-coding antisense transcript of BACE, the enzyme responsible for cleaving APP to generate the toxic A β 42 peptides (Faghihi et al., 2008). This antisense transcript is up-regulated by A β 42, and stabilizes BACE sense transcripts, thereby conferring a feed-forward mechanism to increase A β 42 production (Faghihi et al., 2008). In HD, there is decreased expression of the lincRNA human accelerated region 1 (HAR1), which plays roles in mRNA splicing (Gupta et al., 2010). HAR1 is under the transcriptional regulation of REST – the transcriptional inhibitor which dissociates from mutant huntingtin to translocate into the nucleus in disease states (Johnson et al., 2010). In addition to the dysregulated miRNA pathways due to aberrant REST control, HAR1 may mediate splicing abnormalities in Huntington's disease. Given the potentially broad roles of lincRNAs in diseases like cancer (Gupta et al., 2010) and drug addiction (Michelhaugh et al., 2011), it seems likely that these early examples represent the tip of the iceberg regarding this class of molecules in neuronal death and neurodegenerative disease.

In conjunction with lincRNAs, another intriguing family of non-coding genomic sequences is long interspersed nuclear elements (LINE1 or L1). L1 elements modulate expression of coding genes by retrotransposition. This effect has been seen to alter neural precursors in rat hippocampal stem cells, and affect cell fate choices (Muotri et al., 2005). Furthermore, active retrotransposition may happen in adult animals and lead to heterogeneous and mosaic gene expression profiles (Muotri et al., 2005; Coufal et al., 2009). In addition to altering neurodevelopment, data demonstrate LINES may also be regulated by activity; mice given access to voluntary exercise were seen to have more new LINE1 insertions in subgranular layer neural stem cells than in controls (Muotri et al., 2009). Whether these transposition events serve a meaningful purpose or are perhaps harmful remains to be elucidated, but it is tempting to speculate a role for L1 elements in maintaining genomic flexibility in adult animals. LINE retrotransposition has also been evaluated in the context of neurological dysfunction; mutations in methyl-CpG-binding protein 2 (MeCP2) which cause the neurodevelopmental disorder Rett's Syndrome, lead to an increased incidence of LINE1 retrotransposition (Muotri et al., 2010). However, what impact LINE retrotransposition has in the context of neurodegenerative disease is unknown. As transcriptional and translational control of toxic RNAs and aberrant proteins becomes increasingly paramount to pathology in neurodegeneration, investigating lincRNAs and LINES may prove to be fruitful future avenues of research.

7. Concluding remarks

This review has addressed but a few of the myriad of ways in which RNA and RNA processing might contribute to the pathogenesis of neurodegenerative disease. mRNAs that contain nucleotide repeat sequences can directly elicit neuronal dysfunction by binding to and sequestering critical proteins, preventing them from their normal functions. RNA binding proteins themselves, such as TDP-43 and FUS/TLS, can be mutated or inappropriately localized within neurons and thus contribute to neurodegeneration. Moreover, alterations in the expression and activity of microRNAs and other noncoding RNAs are a common finding in neurodegenerative disease, where they contribute to the pathogenic process in important, though still largely undefined ways.

Yet these lead findings likely represent only the tip of a larger iceberg. Emerging evidence suggests a convergence of pathogenic mechanisms in otherwise disparate neurodegenerative diseases. For example, mutations in TDP-43 have recently been associated with isolated Parkinson's disease phenotypes, with the same mutation leading to ALS in other family members (Quadri et al., 2011). Similarly, "gray zone" CGG repeat expansions (between 40 and 55 CGGs) in the *FMR1* gene are associated with an increased risk of parkinsonism (Hall et al., 2009, 2010, 2011; Loesch et al., 2009, 2011), and non-penetrant CAG repeat expansions in Ataxin-2 (the gene associated with the polyglutamine disorder SCA2) have been identified as a risk factor for ALS and Progressive Supra-nuclear Palsy (Elden et al., 2010; Ross et al., 2011). Perhaps most provocatively, a very recent study identifies a dominantly inherited intronic GGGGCC hexanucleotide repeat expansion in the C9ORF72 gene as a most common inherited cause of both ALS and FTLN (DeJesus-Hernandez et al., 2011; Renton et al., 2011). Although the mechanisms by which these genetic variations contribute to different disease phenotypes are areas of active study, it seems likely that altered RNA networks and RNA processing will play a role.

As next generation sequencing technologies promise ever greater insights into how coding and noncoding RNA networks function, we anticipate significant advances in our understanding of RNA's roles in neuronal function and neurodegenerative disease in the coming years. It is hoped that such advances in our knowledge base will translate to improved therapeutic options for patients. Indeed, preclinical trials of such therapies are beginning to emerge. In myotonic dystrophy, where a CUG mRNA repeat drives disease pathogenesis, morpholino and antisense oligonucleotide based therapies have shown promise in animal models (Mulders et al., 2009; Wheeler et al., 2009). Small molecules designed to block the interactions of the CUG RNA with associated RNA binding proteins are also able to suppress some molecular aspects of the disease phenotype in mice (Pushechnikov et al., 2009; Warf et al., 2009). Similarly, a small peptide screen recently identified a compound capable of interfering with CUG RNA hairpin formation, and its expression or delivery suppressed toxicity in flies and mice (Khateb et al., 2007; Ofer et al., 2009; Pushechnikov et al., 2009). The application of similar approaches to other nucleotide repeat disorders has been proposed and some are already underway (Khateb et al., 2007; Ofer et al., 2009; Pushechnikov et al., 2009). In ALS, the identification of TDP-43 and FUS/TLS as critical proteins in disease pathogenesis have not yet led to targeted compounds in clinical or pre-clinical trials. However, the emergence of new animal models based on mutated forms of these proteins promise to serve as valuable tools for biomarker identification and drug development (Zinman and Cudkowicz, 2011). Lastly, there are emerging efforts to therapeutically target alterations in miRNAs and other noncoding RNAs in neurodegeneration (Eacker et al., 2009; Lau and de Strooper, 2010). While these efforts are largely in their infancy, as our understanding of these RNA networks improves, so should our potential to effectively intervene and correct abnormalities. Thus, although significant work lies ahead, there is great hope that manipulating the activity and expression

of noncoding RNAs will provide a powerful toolset with which to treat neurodegenerative disease.

Acknowledgments

This work was supported by NIH K08NS069809 and the Bucky and Patti Harris Professorship to PK Todd and the Systems and Integrative Biology Training Grant to AJ Renoux.

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Abbreviations

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
Aβ42	amyloid β -42 amino acids
CUGBP1	CUG binding protein 1
DM1	myotonic dystrophy type 1
DM2	myotonic dystrophy type 2
FTD	frontotemporal dementia
FTLD	frontotemporal lobar dementia
FUS/TLS	Fused in Sarcoma/ Translocated in Liposarcoma protein
FXS	Fragile X Syndrome
FXTAS	Fragile X-associated Tremor/Ataxia Syndrome
HAR1	human accelerated region 1
HD	Huntington's disease
HDL2	Huntington disease-like 2
hnRNP	heterogeneous nuclear ribonucleoprotein particles
HSP70	heat shock protein 70
lincRNA	large intergenic non-coding RNA
LINE	long interspersed nuclear element
lncRNA	long non-coding RNA
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MBNL	muscleblind-like
MeCP2	methyl-CpG-binding protein 2
miRNA	microRNA
mRNA	messenger RNA
NEAT1	nuclear enriched abundant transcript 1
NLS	nuclear localization signal
PD	Parkinson's disease
RAN	repeat-associated non-ATG
REST	RE1-silencing transcription factor
SCA10	spinocerebellar ataxia type 10

SCA8	spinocerebellar ataxia type 8
SFRS	serine-rich splicing factors
TDP-43	Tar DNA binding protein of 43 kD
UTR	untranslated region

Fig. 1.

Mechanisms underlying RNA mediated neurodegeneration in nucleotide repeat disorders. Bidirectional transcription at loci with expanded nucleotide repeats leads to the generation of multiple potentially toxic products. (A) Sense transcripts of tri-nucleotide CUG (shown here), CAG, or CGG repeats form secondary mRNA hairpin structures that can bind to and sequester RNA-binding proteins. (B and C) The decreased availability of RNA binding proteins such as splicing factors leads to alterations in the splicing and expression of other mRNAs in trans. Transcription factors and other nucleotide associated proteins may also be sequestered with effects on neuronal homeostasis. (D) Antisense transcription through the repeats leads to the generation of other potentially toxic mRNA sequences. These antisense transcripts sometimes contain open reading frames through the repeat, leading to translation of potentially toxic amino acid homopolymer production (e.g. polyglutamine containing proteins). (E) Homopolymer containing proteins may also be generated through a novel process known as repeat-associated non-ATG initiated (RAN) translation. Shown is the production of a polyglutamine peptide; however, alternative homopolymers in all three potential reading frames may be generated from each transcript.

Fig. 2.

TDP-43 alterations in ALS and other neurodegenerative disorders. (A) TDP-43 is a nuclear protein which associates with the RNA polymerase Pol II on target DNA sequences, and participates in mRNA splicing. (B) Some TDP-43 is also exported from the nucleus associated with RNA-binding proteins on a subset of mRNAs. (C) Chronic neuronal stressors or mutations in TDP-43 lead to the translocation of the majority of TDP-43 to the cytoplasm. (D) This results in a loss of TDP-43's normal nuclear functions related to splicing and transcriptional regulation. (E) The increase in cytoplasmic TDP-43 results in a toxic gain-of-function via generation of TDP-43 protein aggregates as well as the accumulation of TDP-43 in cytoplasmic stress granules.

Table 1

Potentially sequestered proteins in RNA dominant diseases.

Disease	Repeat (antisense)	Bidirectional transcription?	Sequestered protein	Other pathogenesis factors
DM1	CUG (CAG)	Yes	Muscleblind	<ul style="list-style-type: none"> • CUGBP1 activation • RAN translation
DM2	CCUG	Unknown	Muscleblind	<ul style="list-style-type: none"> • ZNF9 insufficiency • Altered protein synthesis
SCA8	CUG (CAG)	Yes	Muscleblind	<ul style="list-style-type: none"> • Antisense encoded polyglutamine • RAN translation
HDL2	CUG (CAG)	Yes	Muscleblind	<ul style="list-style-type: none"> • Antisense encoded polyglutamine
SCA10	AUUCU	Unknown	hnRNP K	<ul style="list-style-type: none"> • Mitochondrial dysfunction
SCA31	UGGAA	Unknown	SFRS1 and 9	<ul style="list-style-type: none"> • Alternate repeat insertion sites
FXTAS	CGG (CCG)	Yes	Muscleblind, hnRNPA2/B1, Pur α , Sam68, lamin A/C, others	<ul style="list-style-type: none"> • Antisense transcript • Mitochondrial dysfunction • FMRP insufficiency • RAN translation?
c9FTD/ALS	GGGGCC	Unknown	Unknown	<ul style="list-style-type: none"> • Altered transcription start site