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RNA-Binding Proteins in Neurodegenerative Disease: TDP-43 and Beyond

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Abstract

Neurodegenerative diseases are a diverse group of disorders that affect different neuron populations, differ in onset and severity, and can be either inherited or sporadic. One common pathological feature of most of these diseases is the presence of insoluble inclusions in and around neurons, which largely consist of misfolded and aggregated protein. For this reason, neurodegenerative diseases are typically thought to be disorders of aberrant protein processing, in which the cumulative effects of misfolded protein aggregates overwhelm the neuron's proteostatic capacity. However, a growing body of evidence suggests a role for abnormal RNA processing in neurodegenerative disease. The importance of RNA metabolism in disease was highlighted by the discovery of TDP-43 (TAR DNA-binding protein), an RNA-binding protein (RBP), as a primary component of insoluble aggregates in patients with sporadic amyotrophic lateral sclerosis (ALS). Subsequently, inherited mutations in TDP-43 and the structurally related RBP, FUS/TLS (fused in sarcoma/translated in liposarcoma), were found to cause ALS. These exciting findings have ushered in a new era of ALS research in which the deregulation of RNA metabolism is viewed as a central cause of motor neuron deterioration. In addition, the fact that neuropathologically and anatomically distinct neurodegenerative diseases display altered RNA metabolism suggests that common pathologic mechanisms may underlie many of these disorders.

Keywords

Neurodegeneration; amyotrophic lateral sclerosis; ribonucleoprotein; aggregation; splicing; TDP-43; FUS; SMN; ataxin

Introduction

Alterations in neuronal RNA processing are characteristic of many if not all neurodegenerative disease states. In many cases, the contribution of RNA alterations to disease is not clear; however, it is increasingly recognized that RNA metabolic abnormalities are capable of directly initiating the neurodegenerative disease process and/or accelerating its progression. In these diseases, inherited mutations lead to disrupted function of RNA binding proteins (RBPs) and subsequent deleterious changes in RNA metabolism.

Recent breakthroughs in understanding the genetic causes of ALS have revolutionized thinking about this disease and have reinforced the concept that inherited or acquired RNA metabolism defects are a common denominator linking diverse neurodegenerative conditions. For the purposes of this review, we shall consider the ALS findings separate

from other neurodegenerative diseases in which RNA deregulation is causal for disease. Common mechanistic themes linking these diverse neurodegenerative conditions will also be discussed. The reader is further directed to a number of excellent in-depth reviews focusing on individual neurodegenerative conditions surveyed below.

1. RNA-BINDING PROTEIN DYSFUNCTION IN NEUROLOGICAL DISEASE

Inherited mutations leading to neurodegenerative disease can be in genes coding for RBPs and directly affect RBP function; alternatively, expansion of trinucleotide repeats in non-RBP genes leads to the production of abnormal RNA that can then affect RBP function. Loss-of-function of RBPs are found in both types of disease, and the resulting global changes in RNA processing are thought to underlie disease pathogenesis.

1.1 Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive disease characterized by the degeneration of motor neurons in the spinal cord, leading to weakness, muscle atrophy and, in severe cases, death. SMA is one of the leading genetic causes of infant mortality, with an estimated occurrence of 1:10,000 live births. SMA is caused by deletion or mutation of the survival of motor neuron (*SMN1*) gene, which encodes a protein that is critical for the assembly of snRNP (small nuclear ribonucleoprotein) particles. In combination with other factors, snRNPs form the core of the spliceosome complex that processes immature mRNA. SMN1 binds to both snRNAs and heptameric Sm proteins and mediates their assembly into snRNPs. SMN1 also facilitates transport of the complex into the nucleus where the mature spliceosome function^{1,2}. Humans harbor a second SMN gene, *SMN2*, that harbors a single nucleotide mutation that leads to skipping of exon 7 and production of only small quantities of functional SMN2 protein³. Copy number variations in the *SMN2* gene modulate the severity of SMA in *SMN1* mutant individuals, and enhancing SMN2 expression is being explored as a therapeutic option in SMA.

SMA has been modeled in mice, which contain only the *SMN1* gene. Because *SMN1* deletion is lethal in mice, SMA has been modeled via introducing *SMN2* in varying copy number in the *SMN1*^{-/-} background⁴. SMA mice demonstrate altered levels of snRNAs and a reduction in functional snRNP complex assembly associated with large changes in alternative splicing⁵. These findings indicate that loss of function of SMN protein leads to aberrant splicing of RNA, which may contribute to the pathogenesis of SMA. However, the contribution of general splicing defects to the initiation of disease in SMA is still not clear, and it is possible that splicing as well as splicing-independent functions of SMN contribute to neuronal integrity⁶.

1.2 Oculopharyngeal Muscular Dystrophy

Oculopharyngeal Muscular Dystrophy (OPMD) is an adult-onset, autosomal dominant degenerative muscular disease characterized by weakness in the muscles of the eye, face, throat, and limbs, as well as cognitive impairment⁷. OPMD is caused by a GCG repeat expansion in the coding region of the gene *PABPN1* (poly(A)-binding protein N1), which encodes the major nuclear polyA-binding protein⁸. Disease-associated mutations in PABPN1 cause an increase in the length of an amino-terminal polyalanine stretch from 10 to 12–17 residues. Normally, PABPN1 binds to nascent poly(A) tails on target mRNA, and recruits PAPs (poly(A) polymerases) that function to increase the length of the tail and thereby stabilize the mRNA. Mutations in OPMD lead to the formation of PABPN1 intranuclear foci predominantly in muscle and, to a lesser extent, brain^{7,9}. These inclusions contain poly(A) mRNA, as well as the RBPs hnRNP A1 and A/B and PAP, all of which bind directly to RNA as well as PABPN1^{10–12}. The pathogenesis of OPMD therefore likely

involves loss of the normal function of PABPN1 and associated RBPs as well as sequestration of target RNAs. PABPN1 aggregation is promoted by the large ribosomal RNA, and drugs interfering with this process reduce PABPN1 toxicity in a *Drosophila* model of OPMD¹³. Like many dominant proteinopathies, the underlying reason for selective tissue vulnerability, in this case muscle, in OPMD is not clear.

1.3 Myotonic Dystrophy type I and II

Patients with myotonic dystrophy (DM) predominantly exhibit muscle degeneration, though they frequently have severe cognitive impairment as well. DM is part of a group of diseases characterized by repeat expansions in non-coding regions of genes. In contrast to OPMD and the polyglutamine diseases, in which repeat expansions in coding regions lead to the production of pathogenic protein, in DM and related disorders the expanded RNA is thought to be the pathogenic species¹⁴. DM1 is caused by a CTG repeat expansion in the 3' UTR of the gene *DMPK* (myotonic dystrophy protein kinase). Whereas 5–37 CTG repeats are considered normal, DM1 patient can have up to several thousand CTG repeats, which leads to pathologically reduced expression of *DMPK* as well as the upstream gene, *SIX5*¹⁵. DM2 is caused by a CCTG repeat expansion in an intron of the gene *ZNF9* (zinc finger protein 9)¹⁶. In both DM1 and DM2 the expanded RNA forms aberrant foci in the nuclei of affected tissues^{14, 17}. These foci also contain members of the muscleblind (MBNL) family of proteins, which are involved in mRNA splicing. Loss of normal MBNL splicing function due to sequestration in these inclusions is thought to be critical for disease pathogenesis. In support of this hypothesis, overexpression of MBNL in DM mice rescues the degenerative phenotype¹⁸, and MBNL knockout mice display phenotypes consistent with DM¹⁹. Furthermore, specific transcripts have been identified that are alternatively spliced in patients with DM; most of these undergo developmentally regulated changes in splicing, and in DM patients the adult splicing pattern is never activated²⁰. Some of these changes can explain symptoms observed in DM: myotonia due to abnormal splicing of CIC-1 (the muscle chloride channel)²¹ and insulin resistance due to abnormal splicing of the insulin receptor²². Though predominantly studied in muscle, foci containing RNA and MBNL proteins are also observed in the neurons of patients with DM. Alternative splicing of neuron-specific transcripts have similarly been found in DM patient brain, including MAPT, NMDAR1, and APP, suggesting that aberrant splicing contributes to the cognitive impairment in DM as well as the muscle phenotype²³. Therefore, though the causative mutation in DM is not in an RBP gene, changes in RBP function as a result of sequestration in nuclear foci are thought to underlie disease pathogenesis. Interestingly, RBP sequestration by expanded repeat RNA may also be important in polyglutamine disease²⁴.

1.4 Mutations in *FMR1*

Repeat expansions in the gene *FMR1* can lead to disrupted RBP function by two distinct mechanisms, leading to two different disease states.

Fragile X Syndrome—Fragile X Syndrome (FXS) is an X-linked disease caused by CGG repeat expansions in the 5' UTR of the *FMR1* gene²⁵. FXS is the most common genetic cause of mental retardation, with an incidence of ~1:2500. Unlike the other repeat-expansions diseases, in which the expanded RNA or protein is pathogenic, expanded CGG repeats in the 5' region of *FMR1* lead to its transcriptional silencing and loss of the FMRP protein.

FMRP is an RBP that associates with RNA in polyribosomes found in dendrites, and is thought to regulate the local translation required for synaptic plasticity. In support of this idea, one primary feature of patients with FXS is the presence of immature dendritic spines^{26, 27}. FMRP also localizes to RNPs on microtubules and may play a role in the

transport of RNA to the synapse²⁸. In *FMR-1* knockout mice, glutamate-induced transport of mRNA to the synapse was reduced²⁹, and FMRP target RNAs and their proteins were altered in both abundance and localization³⁰. Additionally, FMRP was found to regulate the stability of PSD95 mRNA, a critical regulator of synaptic plasticity³¹. These findings indicate that loss of FMRP in FXS leads to dysregulated RNA transport, translation, and stability.

Fragile X Tremor Ataxia Syndrome—In normal individuals the *FMR1* gene contains 5–50 CGG repeats, whereas those with the full disease have >200. Interestingly, individuals with an intermediate number of repeats, termed an *FMR1* premutation, do not develop FXS. Males with an *FMR1* premutation, however, develop FXTAS, a late-onset disorder characterized by dementia, gait abnormality, and tremor¹⁷. In contrast to FXS, in which the *FMR1* gene is silenced, individuals with FXTAS do produce FMRP protein³². However, the expanded RNA forms nuclear foci similar to those observed in DM; these foci also contain RBPs, specifically Pur- α and hnRNP A2/B1^{33,34}. Sequestration and loss of function of these RBPs seems to be important for disease pathogenesis, as overexpression of either protein rescues disease phenotypes in FXTAS animal models^{33,34}. Similarly, another study found that CGG-expanded RNA can sequentially recruit distinct RBPs, including MBNL, to nuclear foci, leading to changes in alternative splicing in FXTAS patients³⁵.

1.5 Spinocerebellar Ataxia Type II

Spinocerebellar ataxia-type II (SCA2) is an autosomal-dominant neurodegenerative disorder caused by CAG expansions in the *ATXN2* gene, which encodes the RBP ataxin-2 (ATXN2). SCAs are movement disorders with diverse genetic origins that preferentially affect the Purkinje neurons of the cerebellum³⁶. In addition to cerebellum symptoms, patients with SCA2 manifest Parkinson's-like symptoms and spinal cord deterioration. In SCA2, the polyQ tract in ATXN2 is expanded from the normal 22 residues to 34 or more residues³⁷. ATXN2-deficient mice do not develop overt ataxia, whereas ATXN2 transgenic mice develop cerebellar ataxia that correlates with degeneration of Purkinje neurons³⁸. PolyQ-expanded ATXN2 is found in cytosolic aggregates, where it may acquire a toxic gain of function.

The normal cellular functions of ATXN2 are still largely unknown. ATXN2 is localized to the Golgi and ER, and has been implicated in endosomal targeting and actin-cytoskeleton dynamics^{39,40}. ATXN2 associates with polyribosomes on the ER, and is implicated in RNA metabolism by virtue of an LSM-type RNA-binding domain, as well as its association with other RBPs, including the cytosolic PABP and A2BP1/FOX-1, a sequence-specific RBP that participates in pre-mRNA splicing^{41,42}. PolyQ expanded forms of ATXN2 compromise the formation of P-bodies and stress granules (SGs), which function as cytosolic mRNA storage depots in response to stress stimuli that compromise mRNA translation⁴³. Beyond this limited information, there is a great deal of uncertainty as to how polyQ expansion of ATXN2 ultimately causes neurodegeneration. However, as will be elaborated below, new evidence has linked ATXN polyQ expansions to classic ALS-like conditions in humans, and this is likely to illuminate mechanisms of ATXN2 neurotoxicity.

2. RNA BINDING PROTEINS IN ALS

ALS is a neurodegenerative disease that targets the spinal motor neurons that control voluntary movement, and is characterized by rapidly progressive weakness and paralysis. ALS carries a cumulative lifetime risk of 1 in 1,000 and is fatal, leading to respiratory failure within 3–5 years. Approximately 90% of ALS cases are sporadic (sALS) and of unknown etiology, whereas ~10% of cases are classified as familial (fALS) and have a clear genetic cause. Dominant mutations in superoxide dismutase 1 (SOD1) account for up to

20% of fALS cases, which are pathologically and clinically similar to sALS⁴⁴. Mutation in SOD1 is thought to impart an abnormal gain-of-function that is toxic to neurons via cell autonomous and non-autonomous mechanisms⁴⁵.

Recently, Neumann et al. sought to identify novel disease-related proteins in patients with ALS and ubiquitin-positive frontotemporal lobar degeneration (FTLD-U), a pathologically similar disease affecting the cortex. They identified the 43-kDa TAR DNA-binding protein (TDP-43) as a common constituent of cytoplasmic inclusions in both ALS and FTLD-U patients⁴⁶. Predominantly nuclear in normal tissues, in disease TDP-43 is mislocalized to the cytoplasm, ubiquitinated, and hyperphosphorylated. Additionally, inherited mutations in TDP-43 and a related RBP, FUS/TLS (fused in sarcoma/translated in liposarcoma, referred to as FUS), were found to cause familial ALS^{47–53}. Since these discoveries, it has been hypothesized that alterations in RNA processing due to TDP-43 and FUS proteinopathy may underlie disease pathogenesis. Since TDP-43 and FUS are structurally related, and because their respective gene mutations cause virtually indistinguishable ALS phenotypes, the functional properties of the proteins and their emergent disease mechanisms will be considered together.

2.1 Structure and cell biology of TDP-43 and FUS

Dominant mutations in the *TARDBP* gene encoding TDP-43 account for up to 4% of familial ALS cases and *de novo* mutation of TDP-43 causes approximately 1% of sporadic ALS⁵⁴. TDP-43 contains a nuclear localization signal (NLS), a nuclear export signal (NES) and two RNA-recognition motifs (RRM1 and RRM2) (Fig. 1). Of these, RRM1 is essential for RNA binding, whereas RRM2 may mediate interactions with ssDNA and TDP-43 self-association^{55, 56}. TDP-43 shows a clear preference for binding to (UG)_n repeat sequences in target RNA sequences. TDP-43 binds to a minimum of six single-stranded dinucleotide stretches, and binding affinity increases with the number of repeats through the highly conserved phenylalanine residues in RRM1⁵⁵. In addition to the RRM, TDP-43 contains an unstructured carboxyl-terminal Gly-rich domain, in which all but one of the 32 identified ALS mutations occurs (Fig. 2). The Gly-rich domain mediates an ever-growing number of interactions between TDP-43 and proteins implicated in various aspects of RNA splicing and RNA metabolism⁵⁷. This domain also contains a Q/N-rich prion-like element that mediates its aggregation with polyQ aggregates^{58, 59}. Finally, cellular TDP-43 migrates as multiple complexes on size exclusion media, suggesting that distinct roles of TDP-43 may be carried out by different protein-protein interactions⁶⁰.

Mutations in the *FUS* gene have been described in ~4% of familial ALS cases and, more rarely, in cases of apparently sporadic etiology^{49, 53, 61, 62}. FUS is predominantly localized to the nucleus, with established functions in transcription, mRNA splicing and transport, and gene silencing. FUS harbors a single centrally located RRM and a Gly-rich domain, which lies just amino-terminal to the RRM. FUS also contains an amino-terminal ~160-amino acid S/Y/Q/G domain rich in Ser, Tyr, Gln, and Gly amino acids, three RGG domains rich in Arg and Gly residues that are also implicated in RNA binding, and a zinc finger domain that binds to GGUG RNA sequences^{63, 64} (Fig. 1). The vast majority of ALS associated mutations in FUS are missense substitutions occurring in the Gly-rich, RGG, or NLS motifs (Fig. 2).

2.2 Normal functions of TDP-43 and FUS

Transcriptional regulation—TDP-43 was originally identified as a transcriptional repressor of the HIV-1 genome, via binding of its RRM to double-stranded HIV TAR DNA repeats. TDP-43 binds to the polypyrimidine-rich region of the TAR DNA and inhibits recruitment of TBP (TATA-binding protein) to TATA elements in both basal and Tat-

induced gene expression⁶⁵. Subsequently, TDP-43 was found to similarly regulate transcription in mammals. The best-characterized example occurs during spermatogenesis, where TDP-43 mediates transcriptional repression of the gene encoding the SP-10 acrosomal protein in round spermatids⁶⁶. FUS has been found in complex with RNA Polymerase II (RNAPII), TFIID, and with several transcription factors, including, PU.1, YB-1, and NF- κ B^{67–70}. FUS generally represses gene expression, though the mechanisms may be diverse. Tan et al. demonstrated that FUS represses expression of RNAPIII, potentially via association with the TATA-binding protein⁷¹. In contrast, FUS negatively regulates cyclin D1 expression by a very different mechanism. In response to DNA damage, FUS binds to single stranded non-coding RNA (ncRNA) transcripts from the cyclin D1 promoter. FUS then binds to and inhibits the histone acetyltransferase activity of the transcriptional coactivator, CREB-binding protein (CBP), leading to Cyclin D1 repression⁷². Overall, the relevance of TDP-43 and FUS transcriptional functions to motor neuron phenotypes in ALS is unknown.

Pre-mRNA splicing—TDP-43 and FUS interact with numerous splicing factors, including the serine/arginine-rich spliceosomal protein SC-35^{73, 74}. Interaction between TDP-43 and the spliceosomal machinery is strongly supported by its interaction with hnRNP proteins, as well as the preponderance of such factors in purified TDP-43 complexes⁷⁵. The cystic fibrosis transmembrane conductance regulator (CFTR) was the first identified splicing substrate of TDP-43, which binds specifically to (UG)_n repeat sequences located at the 3'-splice site of intron 8. In cell models, overexpression of TDP-43 increased exon 9 skipping⁷⁶, and TDP-43 depletion using RNA interference resulted in increased exon 9 inclusion⁷⁷. Because the exclusion of exon 9 results in a non-functional CFTR protein, this finding demonstrates a potential deleterious effect of TDP-43 overexpression and/or deregulation through a defined gene target. Intriguingly, the *SMN2* gene is also a splicing target of TDP-43. As discussed above, *SMN2* differs from *SMN1* only by a translationally silent C (in *SMN1*) to T transition at position 6 of exon 7. SMN2 protein fails to compensate for the loss of SMN1 in SMA because the T nucleotide at position 6 causes exon 7 skipping, resulting in an unstable protein (SMN Δ 7) with reduced oligomerization ability⁷⁸. TDP-43 binds the AG-rich splicing enhancer 2 (SE2) element of the SMN2 pre-mRNA to promote exon 7 inclusion⁷⁹. Finally, the human apolipoprotein A-II (*apoA-II*) gene possesses a (GU)₁₆ stretch in intron 2 that mediates TDP-43-dependent skipping of ApoA-II exon 3⁷³. The splicing function of FUS is not as well documented as that of TDP-43; the splicing complex that includes FUS binds indirectly to 5' splice sites and directly to 3' splice sites of pre-mRNA^{80, 81}. FUS has demonstrated splicing activity against an E1A minigene that required the C-terminal RGG2 domain^{82, 83}.

microRNA biogenesis—miRNAs are derived from hairpin structures of long mRNA transcripts and bind to complementary mRNA, destabilizing it and repressing gene expression⁸⁴. TDP-43 and FUS both interact with Drosha, the RNase III involved in pre-miRNA processing⁸⁵. Buratti et al. identified target miRNAs of TDP-43 via microarray following TDP-43 knockdown in cell culture. TDP-43 potentially regulates numerous miRNAs by binding to their sequence and/or precursor elements; two examined in detail by these authors were let-7b and miR-663. Specifically, let-7b and miR-663 expression levels are down- and upregulated by TDP-43 knockdown, respectively. They also found that expression of a number of target transcripts of these miRNAs was altered in TDP-43 knockdown cells⁸⁶. Beyond this, the mechanisms and disease relevance of TDP-43 and FUS participation in miRNA metabolism are unknown.

mRNA stabilization—In several instances, TDP-43 binding to the 3'-UTR of an mRNA has been linked to changes in its stability. mRNAs encoding the low molecular weight

neurofilament (NFL), cyclin-dependent kinase 6 (CDK6), and histone deacetylase 6 (HDAC6) have each been shown to undergo TDP-43-dependent changes in stabilization in cell culture. Although lacking (UG)_n repeats, the predicted fold of the NFL 3'UTR contains multiple stem loop structures possessing (UG) or (UGUG) motifs on the exposed surface. The RRM domains of TDP-43 are required for binding to NFL mRNA and overexpressing TDP-43 in cell lines increased stability of NFL mRNA⁸⁷. On the other hand, the 3' UTR of the CDK6 pre-mRNA contains (UG)_n repeats that antagonize its expression in a TDP-43-dependent manner⁸⁸. Finally, TDP-43 enhanced HDAC6 mRNA stability through binding to the 3'UTR sequences⁸⁹. HDAC6 is implicated in autophagic degradation of aggregated proteins and suppresses neurodegeneration in animal models of SMA, Alzheimer's disease, and Parkinson's disease^{90, 91}. Interestingly, simultaneous knockdown of TDP-43 and FUS resulted in synergistic reduction in HDAC6 expression, suggesting these proteins operate in complex or linearly in RNA metabolic pathways. In fact, FUS interacts with TDP-43 and the complex of TDP-43 and FUS binds to HDAC6 mRNA, regulating HDAC6 expression^{60, 92}. Finally, although deregulation of NFL, CDK6, and HDAC6 can be plausibly linked to compromised motor neuron function, the functional status of these genes in the setting of ALS are unknown.

Transport and Translation—TDP-43 and FUS have also been implicated in regulating transport of mRNA to dendrites and local translation at synapses. Both proteins have been found in RNA-transporting granules in neurons^{93, 94}. Additionally, as will be discussed further below, emerging animal models of ALS demonstrate dendritic morphology defects^{95, 96}. FUS is particularly implicated in regulating local translation, as it has been found in NMDA receptor complexes⁹⁷ and may regulate transcripts important for the synaptic cytoskeleton⁹⁶. The role of TDP-43 is not as well established, though it has been found to interact with key translational proteins⁷⁵. Finally, both TDP-43 and FUS are found in SGs under conditions of cellular stress^{98–101}. SGs contain stalled translation preinitiation complexes including the ribosome, translation factors, and mRNAs, and the role of TDP-43 and FUS in the regulation of these structures is only beginning to be explored.

2.3 Genomic analysis of TDP-43 RNA substrates

At the time of this writing, several published studies have applied variations of RNA cross-linking, immunoprecipitation, and high-throughput sequencing (CLIP-seq) techniques to identify TDP-43 substrates, as well as specific preferred binding sequences, in brain and cell lines^{100, 102–104}. From these studies, it has emerged that TDP-43 binding is enriched in long intronic regions, the 3'-UTR of pre-mRNA, and nuclear non-coding (nc) RNAs. The strong preference of TDP-43 for (UG)_n repeats motifs was confirmed in these studies; however, UG repeats were neither necessary nor sufficient for binding¹⁰⁰. Polymenidou et al. showed that binding of TDP-43 to intronic sequences correlated positively with expression and suggested that this could reflect a role for TDP-43 in suppressing cryptic splice site expression and aberrant splicing. In addition, binding of TDP-43 to deep intronic regions upstream of an alternatively spliced exon promotes its exclusion, whereas binding of TDP-43 to proximal intronic sequences downstream of the alternatively spliced exon promotes its inclusion¹⁰³. Finally, binding of TDP-43 to 3'UTR sequences was enriched in the cytoplasm, which is consistent with the notion that TDP-43 regulates RNA post-splicing events such as stabilization and translation¹⁰³.

These recent genomic studies identified upwards of 7,000 protein-coding RNA substrates for TDP-43. Within this list are many RNAs involved in neuronal development, neuron survival, and synaptic transmission. TDP-43 splicing targets include myocyte enhancer factor 2D (MEF2D), myocardial infarction associated transcript (MIAT), and Bcl-2 interacting mediator of cell death (BIM)¹⁰³. Interestingly, a number of TDP-43 RNA targets

are directly implicated in neurodegeneration. These include FUS as well as the secreted growth factor progranulin, hemizygous mutations in which cause FTL^{105, 106}. TDP-43 binds to introns 6 and 7 and the 3'-UTR of FUS mRNA to enhance its expression, whereas TDP-43 binding to the 3'-UTR of progranulin mRNA was associated with reduced expression¹⁰⁰. Additionally, Tau and ataxin-1 and -2 were also identified as TDP-43 target mRNAs¹⁰³, though the functional implication of these interactions is not yet clear.

Remarkably, TDP-43 also negatively regulates its own expression by binding to the 3' UTR^{100, 103, 107}. Ectopic expression of TDP-43 represses the expression of endogenous TDP-43, potentially through an alternative splicing event that produces a variant TDP-43 mRNA that is eliminated through nonsense mediated mRNA decay¹⁰⁰. However, another report suggested that TDP-43 autoinhibition occurred independent of splicing¹⁰⁷. Disruption of TDP-43 autoinhibition could lead to feed-forward mechanisms of TDP-43 proteinopathy, in which accumulation of aggregated cytosolic TDP-43 reduces TDP-43 splicing, leading to increased cytosolic mRNA translation, and further TDP-43 aggregation. Such a mechanism plausibly explains the nuclear clearing of TDP-43 that is observed in motor neurons of ALS patients and animal models¹⁰⁸. Future studies will likely focus on the role of TDP-43 autoinhibition in ALS, as well as the effects of ALS-associated mutations on TDP-43 splicing activity.

2.4. Regulation and proteostasis of TDP-43 and FUS

There is currently great interest in understanding how post-translational and/or proteostatic mechanisms influence TDP-43 aggregation and the initiation and/or progression of ALS. Two aspects of TDP-43 regulation—phosphorylation and proteolytic cleavage—have garnered interest for their possible contribution to TDP-43 toxicity. TDP-43 is phosphorylated on Ser-409/410 by Casein Kinase (CK) 1 and 2 *in vitro*, and phosphorylation of these sites increases oligomerization of TDP-43^{109, 110}. Intra-neuronal TDP-43 inclusions are extensively phosphorylated on Ser-409/410, though it remains uncertain whether phosphorylation contributes to TDP-43 aggregation *in vivo*. Interestingly, in a *C. elegans* model of TDP-43 proteinopathy, mutation of Ser-409/410 to Ala reduced toxicity, suggesting a role of phosphorylation in disease pathogenesis¹¹¹. Other sites phosphorylated *in vivo* include Ser-379 and Ser-403/404 in the Gly-rich region. Interestingly, phosphorylation of both clusters was detected in FTL¹⁰⁵ and ALS brains^{112, 113}. Given that CK1 phosphorylated TDP-43 *in vitro* on 29 residues, other phosphorylation sites are certain to be phosphorylated *in vivo*¹¹³.

TDP-43 is also subject to proteolytic cleavage in degenerating motor neurons of ALS patients as well as in cell culture, and C-terminal fragments (CTFs) of TDP-43 have received a great deal of attention for possible neurotoxicity. TDP-43 is cleaved by caspase-3 into fragments of 25 kDa and 35 kDa in brains of ALS and FTL¹⁰⁵-U patients^{114–117}. The CTFs of TDP-43 are redistributed to cytoplasm and induce cytoplasmic aggregation and cytotoxicity in cell culture models^{118–122}. However, the significance of TDP-43 CTFs for motor neuron degeneration *in vivo* has not yet been demonstrated, with one study reporting that CTFs were less toxic than full-length TDP-43 in *Drosophila*¹²³.

Ubiquitinated TDP-43 is a constituent of the cytoplasmic inclusion bodies in degenerating ALS motor neurons, and TDP-43 forms ubiquitin-positive cytosolic aggregates when overexpressed in mammalian cell lines. In cell culture studies, TDP-43 is degraded by both the proteasome and autophagosome pathways^{124–126}. Overexpressed TDP-43 colocalizes with Ubiquitin 1, a protein with dual roles in proteasomal and autophagosomal protein degradation¹²⁷. Ubiquitin 1 binds to ubiquitinated TDP-43 via its UBA domain, which is proposed to mediate the autophagosomal and/or proteasomal targeting of TDP-43 aggregates^{127, 128}. Although autophagy has received a great deal of attention as a protective

mechanism in stressed neurons¹²⁹, the contribution of Ubiquitin proteins to clearance of TDP-43 and other protein aggregates in ALS is still uncertain. Finally, one study found that ALS-associated TDP-43 mutants were more stable than wild-type protein, suggesting that alterations in TDP-43 degradation may be important in disease⁹².

2.5 Animal models of TDP-43 and FUS proteinopathies

In vivo models of TDP-43 proteinopathy have recently been developed in order to understand the mechanisms of TDP-43-induced neurodegeneration¹³⁰. In diverse systems, including flies and mice, expression of TDP-43 in neurons leads to locomotor defects and premature death, mimicking key features of the ALS phenotype. TDP-43 phenotypes worsened with age and, somewhat surprisingly, occurred irrespective of TDP-43 mutation status. Animal models of TDP-43 proteinopathy have begun to address important questions about disease mechanism, including the role of cytoplasmic localization and aggregation, as well the modifying effects of ALS-associated mutations.

Cytoplasmic aggregation versus nuclear toxicity—In *Drosophila*, expression of wild-type TDP-43 specifically in motor neurons was toxic; interestingly, the majority of TDP-43 protein was found in the nucleus, and no cytoplasmic aggregates were detected¹³¹. Furthermore, deletion of the NLS of TDP-43 or mutation of its critical RNA-binding residues rescued the degenerative phenotype¹²³. While these findings suggest a predominantly nuclear toxicity, another study found that deletion of either the NLS or NES of exogenous TDP-43 in *Drosophila* was less toxic than full-length protein, implying a toxic function of both cytoplasmic and nuclear TDP-43¹³². TDP-43 toxicity has also been modeled in *C. elegans*. Expression of wild-type TDP-43 led to an uncoordinated phenotype in worms, which could be abrogated by deletion of either RRM1 or RRM2. In this model, the full-length toxic protein was also nuclear¹³³.

In the first reported transgenic mouse model of TDP-43 proteinopathy, expression of the human A315T mutant TDP-43 led to progressive neurodegeneration and premature death in the absence of cytoplasmic TDP-43 protein aggregates¹³⁴. Interestingly, these mice did have cytoplasmic, ubiquitin positive aggregates in motor neurons; however, they were TDP-43 negative. A more recent study compared transgenic mice expressing wild-type TDP-43 to those expressing NLS-deleted protein¹⁰⁸. Both wild-type and Δ NLS TDP-43 led to neurodegeneration *in vivo*, and in neither case were cytoplasmic aggregates detected. Interestingly, in both cases, endogenous nuclear mouse TDP-43 was depleted. These authors therefore hypothesize that disruption of normal nuclear TDP-43 function may underlie disease¹⁰⁸. Nuclear localization of TDP-43 leading to neurotoxicity has also been observed in rats infected with TDP-43 expressing adenovirus¹³⁵.

In contrast, other groups have reported finding cytoplasmic aggregates in transgenic mouse models of TDP-43 proteinopathy. Interestingly, one group found both cytoplasmic and nuclear aggregates when overexpressing wild-type TDP-43. These aggregates were hyperphosphorylated and ubiquitylated, and contained cleaved TDP-43, similar to the human disease¹³⁶. The presence of both cytoplasmic and nuclear aggregates was also replicated in another model¹³⁷. Similarly, wild-type TDP-43 was recently also expressed specifically in the forebrain of mice to more closely model FTLT; in these mice TDP-43 formed cytoplasmic inclusions and the endogenous nuclear TDP-43 was also depleted¹³⁸. Therefore, while evidence suggests that nuclear localization and RNA binding are critical for disease, cytoplasmic TDP-43 may also exert toxicity *in vivo*.

Relative Toxicity of ALS-Associated Mutations—In the model systems discussed above, both wild-type and mutant TDP-43 are toxic when expressed *in vivo*^{134, 136}. The fact

that wild-type protein is sufficient to induce neurodegeneration, and the observation that wild-type TDP-43 is found in aggregates in sporadic ALS, raises the question of the effect of ALS-associated mutations on disease phenotypes. Results from animal models are informative but have not yet completely addressed this issue. One group compared wild-type and M337V-TDP-43 overexpressing rats, and found that the mutant exhibited more robust neurodegeneration¹³⁹. Similarly, in zebrafish and *C. elegans* mutant TDP-43 was more toxic than wild-type^{111, 140}. However, another group found that wild-type, A315T, and M337V mice all undergo neurodegeneration that was more dependent on expression level than on disease mutation¹⁴¹. In *Drosophila*, two groups have reported that disease mutations actually lead to less severe phenotypes as compared to wild-type protein^{123, 142}. Therefore, it is not yet clear what effect ALS-associated mutations have on disease phenotypes.

Role of Endogenous TDP-43—As noted above, overexpression of human TDP-43 *in vivo* leads to reduction of the endogenous protein level^{108, 138}, likely through regulation of its own mRNA. This raises the possibility that the neurodegeneration observed in animal models is due to depletion and loss of function of the endogenous protein, rather than gain of function of the exogenous TDP-43. Complete knockout of TDP-43 in mice is embryonic lethal^{143, 144}; however, TDP-43 hemizygote mice exhibit motor defects similar to transgenic animals¹⁴⁵. Additionally, in both flies and zebrafish, knockdown or mutation of endogenous TDP-43 homologues leads to severe motor neuron phenotypes that can be rescued by expression of wild-type human TDP-43^{95, 140, 146}. Furthermore, in *Drosophila*, it was found that synaptic bouton morphology alterations caused by loss of the TDP-43 homologue was likely due to altered regulation of a specific TDP-43 RNA target encoding the microtubule associated protein Futsch¹⁴⁷. These findings suggest that loss of function of TDP-43 is sufficient to induce neurodegeneration and motor defects, and further supports the hypothesis that dysregulated nuclear processes may underlie TDP-43 proteinopathy.

FUS Animal Models—FUS-deficient mice die perinatally and exhibit dendritic spine defects, compatible with FUS involvement in important neuronal processes⁹⁶. Wild-type human FUS protein is only mildly toxic when overexpressed in *Drosophila*, while ALS-associated FUS mutants caused robust neurodegeneration^{148, 149}. Mutant FUS also displayed greater cytoplasmic localization as compared to wild-type protein, and deletion of the NES rescued the degenerative phenotype. Interestingly, coexpression of mutant FUS and TDP-43 led to synergistic toxicity, suggesting common pathways may be affected by both proteins¹⁴⁸. Consistent with this, FUS and TDP-43 colocalized when simultaneously expressed in yeast¹⁵⁰. In the yeast model, cytosolic aggregation is strongly implicated in FUS toxicity^{150–153}, and suppressor screens have implicated SG proteins and RNA metabolic pathways as potential mediators of FUS toxicity in this system^{151, 152}. Finally, a recent study examined FUS transgenic rats, which exhibited ALS-like paralysis and neurodegeneration; in this study, mutant FUS protein was more toxic than wild-type¹⁵⁴. All told, the available evidence strongly implicates cytosolic aggregation of FUS as an initiating event in neurodegeneration.

2.5. Other ALS-associated RBPs

Although TDP-43 and FUS have received the greatest notoriety, they are not the only RBPs with clear links to ALS. A slowly progressive, non-fatal, form of ALS with typically juvenile onset (ALS4) is caused by dominant mutations in the SETX gene encoding the DNA/RNA helicase, Senataxin¹⁵⁵. Recent studies indicate that SETX participates in DNA repair and resolves DNA/RNA hybrid structures arising at transcription termination sites, though the relationship of this function to the pathophysiology of ALS4 is unclear^{156, 157}. Interestingly, loss-of-function mutations in SETX also cause the inherited ataxia, AOA2

(ataxia oculomotor apraxia 2), which further supports the concept that many neurodegenerative entities are spectrum diseases ¹⁵⁸.

More recently, Elden et al. reported that intermediate length polyQ expansions in ATXN2 are associated with increased risk for developing ALS ¹⁵⁹. Interestingly, expression of polyQ-expanded ATXN2 worsened TDP-43-dependent neurodegeneration in flies, and TDP-43 and ATXN2 interacted in an RNA-dependent manner in mammalian cells. Additionally, in ALS patients, ATXN2 was mislocalized, and in SCA2 patients, TDP-43 was mislocalized ¹⁵⁹. A screen for ATXN2 expansions in other neurodegenerative diseases identified such expansions in progressive supranuclear palsy (PSP), considered mainly a tauopathy ¹⁶⁰. Combined with the observations that TDP-43 pathology is present in diseases besides ALS and that TDP-43 protein regulates the RNA of disease-related genes, these findings indicate that SCA2, ALS, and other neurodegenerative diseases are related conditions that may share overlapping pathogenic mechanisms.

3. UNIFYING MECHANISMS OF DISEASE PATHOGENESIS

The fact that diverse mutations and dysfunction of RBPs cause different neurological diseases suggests that there may be common perturbations in RNA processing that underlie diverse diseases states. One possibility is that certain critical RNAs are disrupted across diseases, either by alterations in splicing, stability, or translation. This idea is supported by the fact that many of the RBPs implicated in disease likely interact in RNA processing complexes; for example, TDP-43 binds FUS as well as PABPN1 ⁶⁰. However, it seems unlikely that alterations in a few target RNAs are responsible for disease pathogenesis; due to the importance of RBPs in RNA metabolism, it is likely that changes in most of the RNAs within neurons contribute to the development of disease.

Additionally, there is great interest in the causes of TDP-43 and FUS aggregation in motor neurons and how this process contributes to disease pathogenesis. In the case of FUS, there is a compelling reason to suspect a role for ALS mutations in promoting nuclear accumulation and aggregation ¹⁶¹. FUS harbors a non-canonical PY-type NLS near its C-terminus that mediates transportin-dependent nuclear import ^{162, 163}. ALS-associated mutations proximal to the NLS cause abnormal cytosolic accumulation and aggregation of FUS, which recapitulates the phenotype in ALS patients. These findings strongly imply that deregulation of FUS nucleocytoplasmic shuttling is one route whereby FUS elicits motor neuron toxicity. On the other hand, the disease relevance of TDP-43 cytosolic aggregation in ALS is currently less certain, especially in light of the inconsistent findings regarding its occurrence in transgenic animal models. Nonetheless, given the presence of TDP-43 pathology in both familial and sporadic ALS as well as in increasing number of other neurodegenerative conditions, including FTL-D-U and AD ^{164, 165}, it seems likely that TDP-43 cytosolic aggregation plays a role in disease initiation or progression. This idea is supported by findings in yeast, where ALS mutant TDP-43 displays enhanced aggregation and greater toxicity ¹⁶⁶, and a recent study showing prion-like aggregation of the A315T mutant of TDP-43 in mammalian cells ¹⁶⁷. One possibility is that TDP-43 aggregates are directly toxic to neurons, potentially by interfering with proteostatic mechanisms. However, it is also possible that cytoplasmic aggregation of TDP-43 is indirectly toxic via loss of nuclear TDP-43 and its associated transcriptional and RNA processing functions (Fig. 3). This mechanism is supported by the observation that both ALS patients and animal models display loss of nuclear TDP-43. Finally, it is important to account for the fact that the pathology observed in patients is necessarily post-mortem, representing the end stage of disease; hence, it is not known whether aggregation is found early in disease, which would suggest causation. Similarly, in patients with ALS, the motor neurons displaying TDP-43 pathology are necessarily those that have not undergone complete degeneration. It is

therefore possible that TDP-43 aggregation represents a protective feature of the surviving cells. In sum, although the structural relatedness of TDP-43 and FUS would seem to imply that ALS-associated mutations in these proteins elicit similar functional consequences (such as increased aggregation); this may not necessarily be true. Future studies will assuredly resolve the functional similarities and functional differences conferred by ALS-associated mutations in TDP-43 and FUS.

TDP-43 pathology in other neurodegenerative conditions—It is increasingly clear that TDP-43 pathology is common feature of other neurodegenerative conditions, raising the possibility that TDP-43 aggregation is a response to, rather than as a cause of, neuronal stress. In addition to FTLTDP, where TDP-43 aggregation is a near universal finding, TDP-43 aggregates are observed in inclusion body myopathy with Paget's Disease, which is caused by mutations in the *VCP* gene¹⁶⁵. Remarkably, mutations in *VCP*, which mediates both autophagosomal and proteasomal substrate degradation, were recently identified in fALS, suggesting that TDP-43 aggregation in this instance is directly related to the disease process¹⁶⁸. TDP-43 pathology is also observed in up to 50% of Alzheimer's Disease brains and less frequently in Parkinson's Disease and other Lewy body conditions¹⁶⁴. TDP-43 also colocalizes with Htt inclusions in Huntington's Disease¹⁶⁵. The pathologic significance of TDP-43 aggregation in these neurodegenerative conditions is currently unclear.

Conclusion

There is little question that the hunt for initiating pathogenetic mechanisms in ALS is much closer to its prey than it was just five years ago, and excitement in the field is justified given the recent breakthroughs. Nevertheless, as the search for key disease pathways continues, it is important to consider that there are surely multiple steps to the neurodegenerative disease process, which potentially includes nuclear dysfunction as well as cytoplasmic aggregation. One intriguing possibility is that mutations in TDP-43, FUS, and other ALS proteins, in combination with environmental stress, lead to dysregulation of cellular processes, such as RNA metabolism. Over the lifetime of an individual, these stresses lead to progressive neuron dysfunction, culminating in the development of cytoplasmic aggregates containing TDP-43, FUS, and other proteins (Fig. 3). These aggregates may then further the disease process, or represent attempts by the cell to sequester toxic proteins. Regardless of the exact mechanism, which may vary depending on the initiating mutation and combination of other stressors, progressive dysregulation and dysfunction of neurons over an individual's lifetime ultimately leads to cell death and development of disease.

Going forward, it will be important to ascertain the potential cause-and-effect relationships between TDP-43/FUS dysregulation and other pathologic processes associated with motor neuron degeneration. Calcium deregulation and excitotoxicity, long recognized for its contribution to ALS, is one such process¹⁶⁹. Intriguingly, TDP-43 regulates many neuronal calcium pathway mRNAs and TDP-43 SG accumulation is promoted by perturbations in calcium concentration^{98, 100}. Thus, understanding how TDP-43 regulates, and is regulated by, calcium pathway is likely to be an area of future investigation. Finally, the armamentarium of recently developed animal models, combined with genetic and small molecule screening strategies, will no doubt be instrumental in understanding and ultimately combating ALS, as will a better understanding of the biochemical consequences of TDP-43 mutation. It will also be interesting to see whether mutations in additional RBPs contribute to neurodegenerative diseases, including the majority of fALS cases in which a causal gene has yet to be identified. The hope is that these multidisciplinary approaches will ultimately lead to long-sought-after therapeutic breakthroughs for ALS and related neurodegenerative conditions.

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Figure 1.

Comparison of domain architecture in RBPs discussed in this review. Domains in red are known or potential DNA/RNA binding domains. Orange boxes indicate the nuclear localization sequence (NLS); green boxes indicate position of repeats expanded during disease. RRM, RNA recognition motif; ZNF, zinc finger domain; CCD, coiled-coil domain; RNP, ribonucleoprotein-type RNA binding domain; KH, K-homology domain; Lsm, like-Sm domain; LsmAD, Lsm-associated domain; PAM2, PABP-interacting motif 2. Other abbreviations indicate amino acids enriched in indicated domains. Dashes indicate long interdomain regions that have been truncated for clarity.

Figure 2.
ALS-associated mutations in TDP-43 and FUS/TLS (adapted from Dormann et al., Ref. 161).

Figure 3. Speculative mechanism of TDP-43 deregulation and toxicity in ALS. (A) Function and proteostatic regulation of TDP-43 in healthy neurons. Under normal conditions TDP-43 is almost exclusively a nuclear protein, where it participates in pre-mRNA splicing and transcriptional repression. Cytosolic TDP-43 may promote mRNA stability and translation, and is degraded by both proteasomal and autophagosomal pathways. (B) Deregulation of

TDP-43 in pre-symptomatic, vulnerable neurons. Neuronal stressors including TDP-43 mutation, excitotoxicity, ER stress, and alterations in calcium and ATP homeostasis lead to changes in neurons before the onset of gross neurodegeneration. Shuttling of TDP-43 shifts such that more protein is transported to the cytoplasm. TDP-43 protein levels increase due to both reduced degradation and less autoregulation. Increased TDP-43 protein in the cytoplasm leads to changes in processes normally regulated by TDP-43, such as transcription and splicing. Increased cell stress promotes the incorporation of TDP-43 and other proteins into stress granules. (C) TDP-43 deregulation in ALS. In the degenerating neuron, TDP-43 is exclusively in the cytoplasm, where the degradative capacity of the cell is overwhelmed and insoluble aggregates form. Stress granules may nucleate aggregation. TDP-43 autoregulation is reduced further such that even more protein is produced. Loss of TDP-43 in the nucleus and sequestration in aggregates leads to gene expression changes due to the loss of TDP-43 regulation of transcription and splicing.

Table 1

Summary of RBP gene mutations discussed in this review.

Disease	Gene	Inheritance	Mutation	Normal function	Possible disease mechanisms
Spinal Muscular Atrophy	<i>SMN1</i>	autosomal recessive	loss of function	RNA splicing	neuronal splicing defects
Oculopharyngeal Muscular Dystrophy	<i>PABPN1</i>	autosomal dominant	polyalanine expansion of N-terminus	export and shuttling of polyA-mRNA	protein aggregation; loss of normal function; RNA sequestration
Myotonic Dystrophy Type 1	<i>DMPK</i>	autosomal dominant	CTG repeat expansion in 3' UTR	protein kinase, Rho family	toxic RNA; sequestration of MBNL; loss of function
Myotonic Dystrophy Type 2	<i>ZNF9</i>	autosomal dominant	CCTG repeat expansion in intron 1	translation regulation	toxic RNA; sequestration of MBNL; loss of function
Fragile X Syndrome	<i>FMR1</i>	X-linked	>200 CGG repeats in 5' UTR	mRNA transport; local translation	translation defects in dendritic spines
Fragile X Tremor Ataxia Syndrome	<i>FMR1</i>	X-linked	50–200 CGG repeats in 5' UTR	mRNA transport; local translation	toxic RNA; sequestration of splicing factors
Spinocerebellar Ataxia Type II	<i>ATXN2</i>	autosomal dominant	37–50 CAG repeats in exon 1	endosome targeting; actin-cytoskeleton; RNA splicing	protein aggregation; toxic gain of function
ALS10/FTLD-TDP	<i>TARDBP</i>	autosomal dominant	missense mutations in Gly-rich domain	RNA splicing; RNA stabilization; transcriptional repression	protein aggregation; loss of nuclear function; splicing deregulation
ALS6	<i>FUS</i>	autosomal dominant	missense mutations in Gly-rich, RGG, and NLS domains	RNA splicing; transcriptional repression; DNA repair	reduced nuclear import; protein aggregation; splicing deregulation
ALS4	<i>SETX</i>	autosomal dominant	missense/gain of function?	RNA helicase	transcription deregulation
Ataxia Oculomotor Apraxia 2	<i>SETX</i>	autosomal recessive	exon skipping/missense; loss of function	RNA helicase	DNA repair defect; transcription deregulation