

# RNA-Mediated Neuromuscular Disorders

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## Key Words

myotonic dystrophy, fragile X tremor ataxia syndrome, spinocerebellar ataxia, Huntington's disease-like 2

## Abstract

Myotonic dystrophy type 1 (DM1) is caused by a CTG expansion mutation located in the 3' untranslated portion of the dystrophica myotonin protein kinase gene. The identification and characterization of RNA-binding proteins that interact with expanded CUG repeats and the discovery that a similar transcribed but untranslated CCTG expansion in an intron causes myotonic dystrophy type 2 (DM2) have uncovered a new type of mechanism in which microsatellite expansion mutations cause disease through an RNA gain-of-function mechanism. This review discusses RNA pathogenesis in DM1 and DM2 and evidence that similar mechanisms may play a role in a growing number of dominant noncoding expansion disorders, including fragile X tremor ataxia syndrome (FXTAS), spinocerebellar ataxia type 8 (SCA8), SCA10, SCA12, and Huntington's disease-like 2 (HDL2).

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## INTRODUCTION

In general, the initially described trinucleotide expansion diseases fit with a model that microsatellite expansions in noncoding regions (e.g., fragile X mental retardation, Friedreich's ataxia) cause disorders resulting from loss-of-function mechanisms and that dominant disorders with expansions in coding regions (e.g., Huntington's disease, spinocerebellar ataxias) cause disease through gain-of-function mechanisms. In contrast to this trend, myotonic dystrophy type 1 (DM1) was the first example of a dominant disorder caused by a repeat expansion within a noncoding region of a gene (Brook et al. 1992, Fu et al. 1992, Groenen & Wieringa 1998). Almost a decade later, the discovery that a second form of myotonic dystrophy (DM) is caused by a

repeat expansion in an intron of an unrelated second gene, along with other investigations of DM1, has demonstrated that repeat expansions can be pathogenic at the RNA level (Liquori et al. 2001, Ranum & Day 2004). Similarly, substantial evidence now supports that an RNA gain-of-function mechanism may also be involved in the molecular pathogenesis of other noncoding expansion disorders, including fragile X tremor ataxia syndrome (FXTAS), spinocerebellar ataxia type 8 (SCA8), SCA10, SCA12, and Huntington's disease-like 2 (HDL2) (Hagerman & Hagerman 2004; Holmes et al. 2001b, 2003; Lin & Ashizawa 2003; Oostra & Willemssen 2003; Ranum & Day 2004). This review discusses recent developments in the field of dominant noncoding microsatellite expansion disorders, with a focus on RNA gain-of-function mechanisms.

## MYOTONIC DYSTROPHY

The dominantly inherited multisystemic neuromuscular disorders known as the myotonic dystrophies are the best characterized examples of RNA-mediated disease. The mutation causing DM1 is a CTG repeat expansion in the 3' untranslated region (UTR) of the dystrophin myotonia-protein kinase (*DMPK*) gene on chromosome 19 (**Figure 1a**). The length of the CTG expansion in unaffected individuals ranges from 5 to 38, whereas individuals with as few as 50 repeats can show symptoms of the disease. Disease severity and age of onset correlate with repeat length, and repeat expansions of >1500 often result in a severe congenital form of DM1. DM affects multiple organ systems with major symptoms, including progressive skeletal muscle wasting; impaired muscle relaxation (myotonia); cardiac conduction defects resulting in arrhythmias; early-onset iridescent cataracts; insulin resistance and hyperinsulinemia; testicular failure; frontal balding; and altered central nervous system (CNS) function, including cognitive impairment, somnolence, and behavioral disturbances (Harper 2001).

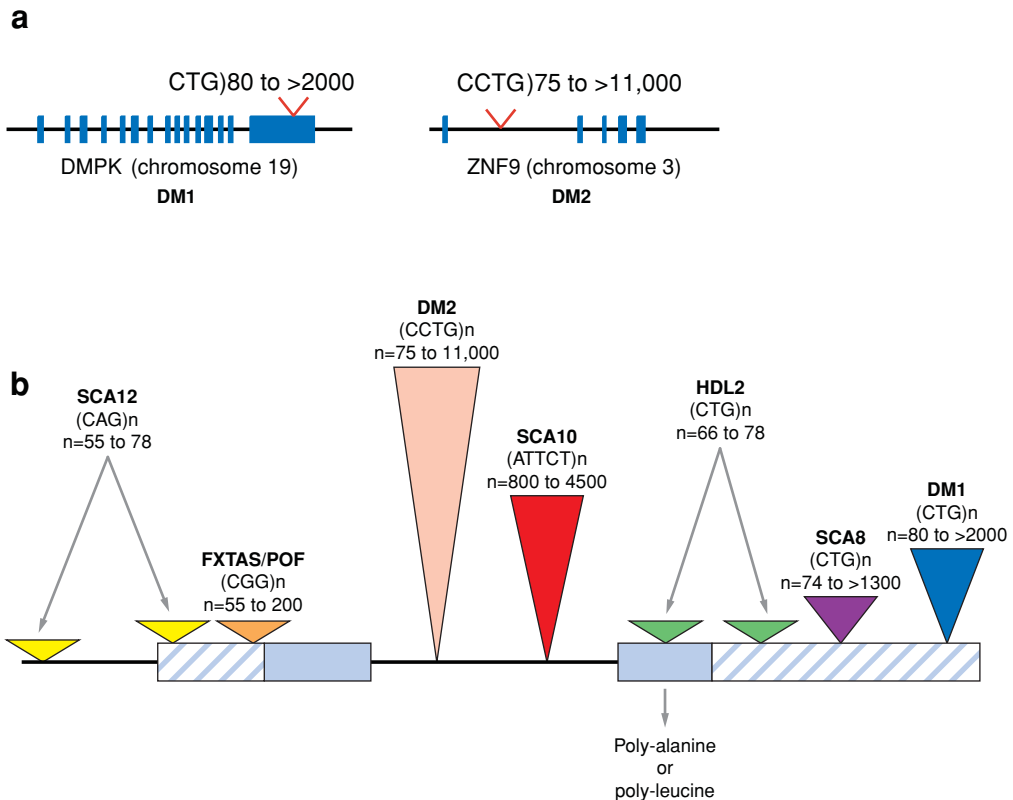
**DM1/DM2:**  
myotonic dystrophy  
type 1/2

**FXTAS:** fragile X  
tremor ataxia  
syndrome

**SCA:**  
spinocerebellar  
ataxia

**HDL2:**  
Huntington's  
disease-like 2

**DMPK:** dystrophin  
myotonic protein  
kinase



**Figure 1**

Mutations causing known and potential RNA gain-of-function effects. (a) CTG and CCTG expansions in the *DMPK* and *ZNF9* genes cause DM1 and DM2, respectively. (b) Noncoding microsatellite expansion disorders. Schematic diagram showing position and relative sizes of disease-associated microsatellite repeat expansions that are located, or can be located, within noncoding portions of their respective genes. Schematic depiction of various regions of theoretical gene: black lines represent promoter (left) and intron (right), shaded blue boxes represent 5' and 3' untranslated regions, and solid blue boxes represent protein coding regions. The locations of the repeat expansions for SCA12 and HDL2 differ depending on the start site or alternative splicing pattern of those genes.

Individuals with a second form of DM have a CCTG tetranucleotide expansion within intron 1 of the *ZNF9* (zinc finger 9) gene on chromosome 3 (Liquori et al. 2001) (**Figure 1a**). Researchers independently identified and described this form of the disease, called myotonic dystrophy type 2 (DM2), as proximal myotonic myopathy (Meola & Moxley 2004, Ricker et al. 1994, Thornton et al. 1994). The expansion in DM2 ranges in size from 75 to 11,000 CCTG repeats. The clinical presentation of DM1 and

DM2 are remarkably similar given that the diseases result from different mutations in different loci, strongly suggesting that the rare constellation of clinical features shared by DM1 and DM2 share a common molecular etiology. However, differences in the clinical presentation are also informative. These differences include the muscle groups most affected (proximal in DM2 and distal in DM1), overall increased severity of the muscular dystrophy and CNS symptoms in DM1, and the absence of a congenital form in DM2.

**ZNF9:** zinc finger 9

## Evidence for an RNA Gain-of-Function Mechanism

The mutation causing DM1 was identified in 1992; however, the realization that RNA from the expanded allele played a major role in the pathogenic mechanism evolved over the course of nearly a decade. That a repeat expansion in a nontranslated region causes a dominantly inherited disease was indicative of either haploinsufficiency of *DMPK*, loci located close to *DMPK*, or a gain of function for the repeat expansion, either as DNA or RNA. Several early pieces of evidence pointed to an RNA gain-of-function mechanism for DM pathogenesis. Mouse *Dmpk* knockout models for a loss of function failed to reproduce the array of features expected of a DM phenotype. Homozygous *Dmpk* knockout mice exhibit progressive defects in cardiac conduction (Berul et al. 2000) and decreased force generation of skeletal muscle (Reddy et al. 1996). However, the effects are relatively mild and do not represent the genetic situation in human disease in which one normal allele is present. Another hypothesis was that the expanded repeat decreased expression of the *SIX5* gene, which is located 1 kb downstream of the *DMPK* locus (Tapscott & Thornton 2001). *Six5*<sup>-/+</sup> mice exhibited only cataracts that were not of the rare posterior iridescent type observed in DM1 (Klesert et al. 2000, Sarkar et al. 2000). Therefore, loss of function of *Dmpk* or *Six5* did not reproduce a DM-like syndrome in mice.

In contrast, several early pieces of evidence supported the unorthodox hypothesis that expression of RNAs from the expanded allele induces the pathogenic effects of the mutation. First, in the important demonstration using in situ hybridization, the expanded allele is transcribed, and the mRNAs are appropriately spliced but nevertheless retained in the nucleus in punctate foci (Davis et al. 1997). Analysis using biochemical fractionation demonstrated that the RNA is tightly associated with nuclear components. Prior reports conflicted as to whether RNA was transcribed from

the expanded *DMPK* allele. However, the use of a cesium chloride ultracentrifugation step provided the high salt necessary to extract the repeat-containing transcripts. Consistent with in situ hybridization studies, cell fractionation studies demonstrated that virtually all of the expanded transcripts are in the nucleus (Davis et al. 1997). Second, the finding that a CCTG expansion in a gene unlinked and unrelated to *DMPK* produced a very similar disease (DM2) strongly indicated that loss of *DMPK* or *SIX5* function was not responsible for the major features of the disease (Liquori et al. 2001). Furthermore, the finding that the CCUG repeat-containing RNA expressed from this gene also form nuclear foci provided strong support for the role of RNA containing the expanded repeats. Third, DM is the most common form of adult-onset muscular dystrophy with a prevalence of 1 in 8500 individuals worldwide. Only CTG or CCTG expansions have been identified in a large number of individuals screened for disease-causing mutations. Furthermore, although a concerted effort was made, no examples of loss-of-function mutations in *DMPK* have been found which strongly suggests that expression of the repeats rather than loss of function of the genes containing the repeats causes this disease. Fourth, mouse models provided direct experimental support for the RNA gain-of-function hypothesis (Mankodi et al. 2000, Seznec et al. 2001). Transgenic lines of mice that express the human skeletal muscle alpha actin gene containing 250 CTG repeats inserted into the actin 3' UTR were generated (Mankodi et al. 2000). These mice (HSA<sup>LR</sup>) expressed large amounts of repeat-containing RNA in skeletal muscle owing to the highly active HSA promoter. The RNA localizes within nuclear foci, and the mice exhibit several features of DM skeletal muscle, including histological features such as sarcoplasmic masses, chains of nuclei centrally located within the myofiber, and ringed fibers. Importantly, the HSA<sup>LR</sup> mice also exhibited myotonia, a prominent skeletal muscle feature of the human disease. In contrast, mice

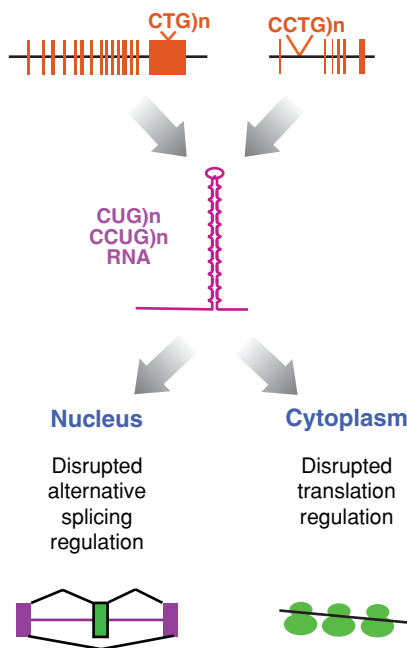
expressing the identical RNA containing five CTG repeats showed no DM-like phenotype (Mankodi et al. 2000). These results indicate that expression of the CTG repeats alone, in the context of a non-*DMPK* mRNA, were sufficient to reproduce at least some of the features of DM in mice.

Seznec et al. (2001) generated a second transgenic mouse model (DM300) containing a large genomic fragment, including an expanded [(CTG)<sub>300</sub>] *DMPK* gene as well as flanking genes. These mice differ from the HSA<sup>LR</sup> mice in that the repeats are expressed within their natural context of the *DMPK* mRNA, and the level of repeat-containing mRNA is likely to be lower because transcription is driven by the *DMPK* promoter. In addition, although expression of repeat-containing mRNA is limited to skeletal muscle in HSA<sup>LR</sup> mice, DM300 mice express repeat-containing RNA in several tissues. These mice exhibit myotonia, skeletal muscle histological abnormalities, and, in the brain, altered expression of tau isoforms, similar to what is observed in individuals with DM1.

Results from several laboratories indicate that nuclear accumulation of CUG and CCUG RNA transcribed from the expanded alleles disrupts both nuclear and cytoplasmic RNA processing events (**Figure 2**). We first describe the effects of an RNA gain of function on nuclear RNA processing (pre-mRNA alternative splicing) and then on regulation of translation in the cytoplasm.

### Alternative Splicing Misregulation in Myotonic Dystrophy

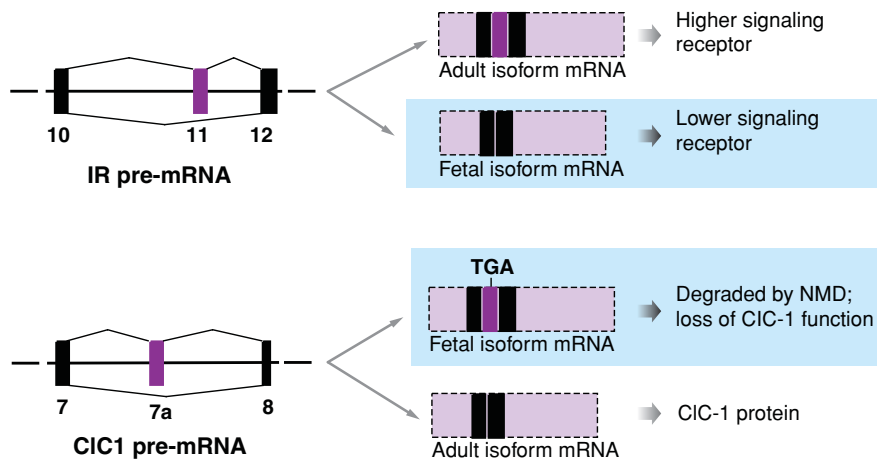
The human genome contains 20,000–25,000 genes that express hundreds of thousands of proteins (Int. Hum. Genome Seq. Consort. 2004). A primary source of proteome diversity is alternative splicing, a process in which a pre-mRNA from an individual gene is processed in different ways to produce multiple mRNAs (**Figure 3**). Up to 74% of human genes express multiple mRNAs via alternative splicing (Johnson et al. 2003), the out-



**Figure 2**

The RNA gain-of-function model of myotonic dystrophy pathogenesis. RNA transcripts expressed from the expanded *DMPK* and *ZNF9* alleles accumulate in the nuclei. The expanded CUG and CCUG RNA form an imperfect double-stranded structure. The nuclear accumulation of this RNA disrupts RNA-processing functions in the nucleus and cytoplasm, affecting the regulation of alternative splicing and translation.

come of which is to create variable segments within mRNAs that are otherwise identical. Eighty percent of this variability is within the open reading frame of the mRNA, resulting in the expression of divergent protein isoforms (Modrek et al. 2001). Differences in the functions of these isoforms can have a significant impact on cell physiology such that regulation of alternative splicing can be determinative for cellular transitions. In addition, up to 30% of alternative splicing events introduce premature termination codons into an mRNA by changing the reading frame and causing degradation of the mRNA by nonsense-mediated decay (Lewis et al. 2003). Therefore, in addition to directing the



**Figure 3**

Misregulation of alternative splicing in DM skeletal muscle. Developmental regulation of IR exon 11 and CIC-1 exon 7a is altered in adult DM skeletal muscle such that the fetal splicing pattern predominates (*gray background*). The fetal splicing pattern of IR results in expression of an isoform with a lower signaling potential than the isoform expressed in adult skeletal muscle. CIC-1 exon 7a contains a premature termination codon resulting in degradation of mRNA by nonsense-mediated decay (NMD). The subsequent loss of CIC-1 protein expression in adult skeletal muscle results in myotonia.

expression of diverse protein isoforms, the control of alternative splicing can direct on-off expression of genes.

The expression of alternatively spliced mRNAs is often regulated according to cell type, developmental stage, or in response to external cues. This regulation involves binding of RNA-binding proteins to specific sites within the pre-mRNA, usually within a few hundred nucleotides of the regulated splice site. The mechanism for cell-specific alternative splicing regulation in most experimental systems examined involves the activities of antagonistic factors that promote different pathways, rather than the activity of one tissue-specific regulatory protein (Black 2003). Therefore, it is the balance between the nuclear activities of different regulators that determines cell-specific regulation.

A predominant molecular feature of DM is the misregulation of alternative splicing. Thirteen alternative splicing events are misregulated in three different tissues from individuals with DM (**Table 1**). All of the misregulated splicing events are normally devel-

opmentally regulated, and in DM, all fail to express the complete adult splicing pattern in adult tissues. For at least two genes, the fetal mRNA and protein isoforms fail to provide the functions required in adult tissues, which results in prominent features of the disease (described in the next paragraph). It is important to distinguish the misregulation of splicing observed in DM from the aberrant splicing identified as causative of many other genetic diseases (Faustino & Cooper 2003). Diseases associated with aberrant splicing are caused by mutations within exons or splice site sequences at the exon-intron borders, which interfere with proper splicing. Such mutations result in either exon skipping or the use of cryptic splice sites and expression of non-natural mRNAs. Most often these mRNAs are out of frame, which results in the loss of function of the mutant allele. In contrast, the splicing defects in DM reflect a loss of appropriate regulation. Natural mRNA variants are expressed; however, these mRNAs are expressed in inappropriate tissues or developmental stages.



**Table 1** Misregulated alternative splicing in myotonic dystrophy

Gene	Exon/Intron	Tissue	DM pattern	References
Cardiac troponin T (TNNT2)	Exon 5	Heart	Exon inclusion	Philips et al. 1998
Insulin receptor (IR)	Exon 11	Skeletal muscle	Exon exclusion	Savkur et al. 2001, 2004
Chloride channel (CLCN-1)	Intron 2	Skeletal muscle	Intron retention	Charlet et al. 2002, Mankodi et al. 2002
Chloride channel (CLCN-1)	Exon 7a	Skeletal muscle	Exon inclusion	Charlet et al. 2002, Mankodi et al. 2002
Tau (MAPT)	Exons 2 and 3	Brain	Exon exclusion	Jiang et al. 2004a, Sergeant et al. 2001
Tau (MAPT)	Exon 10	Brain	Exon exclusion	Jiang et al. 2004b
Myotubularin-related protein 1 (MTMR1)	Exons 2.1 and 2.3	Skeletal muscle/heart	Exon exclusion	Buj-Bello et al. 2002, Ho et al. 2005a
Fast skeletal troponin T (TNNT3)	Fetal exon	Skeletal muscle	Exon inclusion	Kanadia et al. 2003
N-methyl-D-aspartate receptor (NMDAR1)	Exon 5	Brain	Exon inclusion	Jiang et al. 2004a
Amyloid precursor protein (APP)	Exon 7	Brain	Exon exclusion	Jiang et al. 2004a
Ryanodine receptor (RyR)	Exon 70 (AS I)	Skeletal muscle	Exon exclusion	Kimura et al. 2005
Sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> ATPase 1 (SERCA1)	Exon 22	Skeletal muscle	Exon exclusion	Kimura et al. 2005
Sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> ATPase 2 (SERCA2)	Intron 19	Skeletal muscle	Intron retention	Kimura et al. 2005

Of the known misregulated splicing events associated with DM, two correlate directly with disease symptoms: The myotonia and insulin resistance of DM correlates with misregulated splicing of the muscle-specific chloride channel (CIC-1) and insulin receptor (IR), respectively. Myotonia correlates with significantly reduced expression of CIC-1 in both patients and the HSR<sup>LS</sup> mouse model (Charlet et al. 2002, Mankodi et al. 2002). Individuals with DM exhibit reduced expression of CIC-1 protein to less than 10% of normal levels, which is sufficient to cause myotonia in individuals with familial myotonia caused by loss-of-function mutations in the CIC-1 gene. The loss of CIC-1 protein in DM is caused by a failure to switch from an embryonic splicing pattern, in which inclusion of one or both of two alternative exons puts the CIC-1 mRNA out of frame, to the adult splicing pattern in which these exons are skipped (Charlet et al. 2002, Mankodi et al. 2002).

CIC-1 mRNAs that include these exons are likely to be degraded by nonsense-mediated decay. Therefore, the normal developmental regulation of CIC-1 mRNA and protein expression appears to be controlled by an alternative splicing decision that is misregulated in DM skeletal muscle.

DM is associated with an unusual form of insulin resistance owing to a specific defect in skeletal muscle (Moxley et al. 1978). Insulin resistance in DM correlates with a failure to express the IR splice variant that normally predominates in adult skeletal muscle. Individuals with DM express a nonmuscle IR splice variant with a lower signaling capability than the isoform normally expressed in adult skeletal muscle. This isoform also predominates in DM skeletal muscle cultures, and its expression correlates with reduced responsiveness of these cultures to insulin compared with control cultures from unaffected individuals (Savkur et al. 2001). The IR

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**CELF:** CUG-BP and ETR-3-like factors

**MBNL:** muscleblind-like

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splicing abnormalities observed in DM1 have also been found in DM2 skeletal muscle, consistent with the finding that insulin resistance is observed in both forms of the disease (Savkur et al. 2004). For the other misregulated splicing events listed in **Table 1**, there are several possible correlations between the misexpression of an inappropriate isoform and disease symptoms. However, the specific cause-effect relationships remain to be established.

### Mechanism of Splicing Misregulation

CUG and CCUG repeat-containing RNAs clearly have an effect in *trans* and alter the normal splicing patterns of a subset of pre-mRNAs. HSA<sup>LR</sup> mice exhibit altered splicing of *Cic-1* pre-mRNAs, demonstrating the effect of CUG repeats in the context of a non-*DMPK* mRNA (Mankodi et al. 2000). Additional evidence that CUG repeat-containing mRNA induce a splicing switch comes from transient expression of CUG repeat-containing *DMPK* RNA in cell culture. Co-expression of CUG repeat-containing mRNA with splicing reporter minigene constructs of cardiac troponin T (cTNT) and IR induces the DM splicing pattern for these alternative exons (Charlet et al. 2002, Philips et al. 1998, Savkur et al. 2001). The mechanism by which CUG repeat RNA induces a *trans*-dominant effect on splicing regulation is unclear. The most straightforward explanation is that one or more splicing regulators are sequestered on repeat-containing RNA, which results in nuclear depletion and a loss of function of these regulators. In support of this hypothesis, two RNA-binding proteins demonstrated to regulate alternative splicing were identified on the basis of their binding to CUG repeat-containing RNA (Miller et al. 2000, Timchenko et al. 1996). The first to be identified, CUG-BP1, is a member of a family of six genes encoding proteins that regulate several post-transcriptional processing steps, including alternative splicing, RNA editing, translation initiation, and mRNA stability

(Anant et al. 2001, Ladd et al. 2001, Mukhopadhyay et al. 2003, Paillard et al. 1998, Timchenko et al. 1999). This family is called the CUG-BP and ETR-3-like factors (CELF). The second family of CUG repeat-binding proteins, of which there are three genes, are muscleblind-like (MBNL). MBNL proteins, like CELF proteins, directly regulate alternative splicing by binding to specific binding sites within the pre-mRNA.

Several pieces of evidence indicate a role for the loss of MBNL function in splicing misregulation in DM. First, MBNL proteins bind to expanded CUG repeat RNA, which folds into a hairpin structure containing G-C base pairs and bulged, unpaired U residues (Napierala & Krzyzosiak 1997). Significantly, MBNL binding correlates with repeat length (Miller et al. 2000), consistent with the direct correlation between the length of the repeat expansion and disease severity. In addition, MBNL proteins from all three *MBNL* genes colocalize with the nuclear foci of CUG or CCUG repeat-containing RNA, supporting the view that MBNL is sequestered (Fardaei et al. 2002, Mankodi et al. 2003, Miller et al. 2000). Second, a knockout of *Mbnl1* exon 3 in the *Mbnl1*<sup>ΔE3/ΔE3</sup> mouse line prevents expression of the predominant isoforms of the *Mbnl1* gene and causes a DM-like phenotype, including posterior iridescent cataracts, histological changes in skeletal muscle, and misregulated splicing events, all characteristic of DM (Kanadia et al. 2003). In fact, the HSA<sup>LR</sup> and *Mbnl1*<sup>ΔE3/ΔE3</sup> mice exhibit strong similarities in histology and alternative splicing misregulation, demonstrating the link between loss of MBNL function and splicing abnormalities induced by expression of CUG repeat RNA. Third, MBNL proteins can bind directly to intronic elements and regulate the cTNT and IR alternative splicing events that are misregulated in DM (Ho et al. 2004). siRNA depletion of MBNL1 in HeLa cells induces the splicing pattern observed in DM tissue. These results provide a direct link between loss of function of MBNL proteins by sequestration and altered splicing regulation.



Several pieces of evidence also suggest a role for CELF proteins in misregulated splicing of DM, with CUG-BP1 being the most studied family member. First, CUG-BP1 was identified on the basis of its binding to CUG repeat RNA (Timchenko et al. 1996). Unlike MBNL1, CUG-BP1 binds to short single-stranded CUG repeats but does not bind proportionally to the length of the repeat RNA. Neither CUG-BP1 nor other CELF protein family members colocalize with CUG or CCUG repeat RNA foci (Jiang et al. 2004b). Second, all three of the misregulated splicing events observed in DM that were tested (cTNT, IR, and CIC-1) were regulated by CUG-BP1 (Charlet et al. 2002, Philips et al. 1998, Savkur et al. 2001). Third, the splicing patterns observed for all three of these splicing events are consistent with increased CUG-BP1 activity. Fourth, CUG-BP1 protein steady-state levels are increased in tissues and cell cultures from individuals with DM (Dansithong et al. 2005, Savkur et al. 2001, Timchenko et al. 2001). Fifth, CUG-BP1 protein expression normally decreases in mouse skeletal muscle and heart soon after birth (Ladd et al. 2005), consistent with the normal developmental regulation of the splicing events in these tissues that are altered in DM. Transgenic mice overexpressing CUG-BP1 in heart and skeletal muscle result in a return of the fetal splicing pattern for cTNT, CIC-1, and myotubularin-related protein 1 (Ho et al. 2005a). These mice die at birth, most likely owing to skeletal muscle dysfunction and the inability to breathe.

Both MBNL1 and CUG-BP1 can regulate at least two of the splicing events that are misregulated in DM, cTNT, and IR. Importantly, these two proteins are antagonistic regulators of these two splicing events such that the splicing patterns observed in DM are consistent with a loss of MBNL and a gain of CUG-BP1 activities. The evidence cited above strongly supports loss of MBNL activity as the dominant cause of splicing abnormalities in DM, and the role of CUG-BP1 could be the antagonistic regulator that assumes a dominant

role secondary to loss of its antagonist. However, an alternative model proposes that loss of MBNL activity is not sufficient to explain the splicing abnormalities observed in DM. The evidence is based on results from transient transfection assays in which expression of CUG repeat-containing RNA induces a splicing switch in *trans* on co-expressed cTNT and IR minigenes. The splicing of these minigenes serves as reporters of the *trans* effects of co-expressed CUG repeat-containing RNA. Mutations in the CUG-BP1 binding sites that render cTNT and IR minigenes nonresponsive to CUG-BP1 also render them nonresponsive to the *trans* effects of RNA-containing CUG repeats. These results indicate that direct binding of CUG-BP1 to the cTNT and IR pre-mRNAs is required for the co-expressed repeats to affect splicing (Philips et al. 1998, Savkur et al. 2001). In addition, the mutated cTNT minigene that is not responsive to CUG-BP1 or CUG repeat RNA responds to MBNL1 siRNA-mediated depletion as strongly as the nonmutated minigene (Ho et al. 2005a). These results indicate that the co-expressed CUG repeat RNA is not depleting MBNL sufficiently to induce the splicing change observed for the wild-type minigene. If this were the case, then the mutant minigene would also exhibit a splicing response to the repeats because it is competent to respond to MBNL depletion. The effect of the CUG repeat RNA on alternative splicing of the wild-type cTNT minigene involves more than MBNL depletion. It is unclear what this effect may be. However, early results indicated a change in the phosphorylation status and nuclear:cytoplasmic distribution of CUG-BP1 (Roberts et al. 1997). Such changes could affect nuclear protein steady-state levels or intrinsic RNA-binding or splicing activity. Third, in a series of experiments, identical *DMPK* mRNAs containing CUG or CAG expanded repeats were expressed with cTNT and IR minigenes. Only the CUG repeat-containing RNAs had a strong effect on cTNT and IR splicing. However, both CUG and CAG repeat RNA

formed nuclear foci and colocalized with co-expressed GFP-MBNL1. Furthermore, analysis using fluorescence recovery after photobleaching (FRAP) indicated that the *in vivo* affinity of GFPMBNL1 for CAG and CUG repeat foci were indistinguishable (Ho et al. 2005b). These results support the contention that colocalization of MBNL1 with RNA foci is not equivalent to MBNL1 sequestration and that the *trans* effect of CUG repeats on splicing involves more than sequestration of MBNL1.

Therefore, a second, but not mutually exclusive, model for the effects of repeat-containing RNA on splicing proposes that both CELF and MBNL proteins are regulators of a subset of developmentally regulated alternative splicing events. Consistent with this proposal is the loss of CELF protein expression during development of most tissues (Ladd et al. 2005) and the fact that both of two developmentally regulated splicing events examined were found to be antagonistically regulated by CELF and MBNL proteins (Ho et al. 2004). Changes in MBNL and CELF activities can be viewed as downstream effects of signaling events involved in the regulation of developmental splicing transitions. The nuclear accumulation of repeat-containing RNA could affect these signaling events either by artificially stimulating the embryonic program or preventing the transition from embryonic to adult splicing patterns. Identification of the relevant signaling events will provide significant insight into normal regulation of alternative splicing transitions, as well as into the pathogenic mechanisms involved in DM.

### Role for Disruption of Cytoplasmic RNA Processing

RNA-binding proteins that regulate nuclear RNA-processing events such as alternative splicing often regulate RNA-processing events in the cytoplasm as well (Shyu & Wilkinson 2000). CUG-BP1 is both nuclear and cytoplasmic (Roberts et al. 1997,

Timchenko et al. 1999), is associated with polysomes (Timchenko et al. 1999), and can regulate translation of p21, C/EBP $\beta$  LIP, and MEF2A (Timchenko et al. 2001, 2004). Of particular relevance to DM are proteins p21 and MEF2A, which have roles in skeletal muscle differentiation. Cultured DM muscle cells are defective in their ability to withdraw from the cell cycle, a necessary step for muscle differentiation (Timchenko et al. 2001). DM1 muscle cultures exhibited an increase in nuclear CUG-BP1 and a corresponding loss of cytoplasmic CUG-BP1 compared with muscle cultures from unaffected individuals. The loss of cytoplasmic CUG-BP1 correlates with a failure to express p21 protein during differentiation, despite expression of normal levels of p21 mRNA. CUG-BP1 directly enhanced translation of p21 mRNA by binding to (GCN) $_n$  motifs within a 5' coding region (Timchenko et al. 2001). Consistent with the proposal that failure to express p21 in DM cells results in defects in cell cycle withdrawal, cdk4 protein levels were increased in DM cultures compared with normal controls, and there was a failure of DM cells to form Rb-E2F complexes, which are required to inhibit cell cycle progression (Timchenko et al. 2001). Similarly, CUG-BP1 increases translation of *MEF2A* mRNA through direct interaction with CAG repeats near the 3' end of the open reading frame, and the loss of cytoplasmic CUG-BP1 in DM muscle is proposed to reduce *MEF2A*, which promotes muscle differentiation (Timchenko et al. 2004).

### Molecular Differences Between DM1 and DM2 that may Contribute to their Clinical Distinctions

Although adult-onset DM1 and DM2 have similar multisystemic presentations, DM2 is generally milder, without the severe developmental deficits or mental retardation seen in DM1 patients with congenital-onset DM1 (Day et al. 2003). Researchers have proposed that these differences may result partially from differences that the DM1 or DM2 repeat

expansions have on the expression of *DMPK*, *ZNF9*, or neighboring genes, or from different affinities of RNA-binding proteins (e.g., CUG-BP1, MBNL1, or others) for the CUG versus CCUG motifs. Alternatively, a hypothesis that may explain a role for RNA pathogenesis in other disorders is that differences in the temporal and spatial expression patterns of the repeat-containing RNAs in congenital-onset DM1 as well as adult-onset DM1 and DM2 could account for the clinical distinctions between these disorders. Consistent with this hypothesis, Filippova et al. (2001) have shown that CTCF-binding sites that flank the DM1 CTG repeat are capable of acting as insulator elements between *DMPK* and *SIX5*. Their in vitro and cell culture work have suggested the possibility that methylation occurring at the DM1 locus in congenital cases may increase *DMPK* expression by activating the nearby *SIX5* enhancer, a model consistent with the idea that increased expression of CUG expansion transcripts in congenital DM1 may cause the more severe phenotype (Cho et al. 2005, Filippova et al. 2001).

## OTHER DOMINANT NONCODING EXPANSION DISORDERS

In recent years, researchers have identified a growing number of other dominant noncoding microsatellite expansion mutations and, in addition, new problems resulting from the expression of relatively short CGG repeat expansions have been recognized as causes of premature ovarian failure (POF) and fragile X tremor ataxia syndrome (FXTAS). A brief description of these disorders and possible dominant effects of these mutations at the RNA level are discussed below.

### Role of CGG Expansion RNAs in Fragile X Tremor Ataxia Syndrome

In addition to fragile X syndrome, the clinically distinct disorders of POF and FXTAS are also caused by CGG triplet expansions in

the *FMRI* gene (Oostra & Willemsen 2003) (**Figure 1b**). CGG expansions greater than 200 repeats cause fragile X syndrome through a loss-of-function mechanism, whereas smaller “premutation” expansions (55–200 repeats) are associated with POF and FXTAS. There is growing support for a model in which alleles in the premutation range can cause FXTAS and possibly POF through an RNA gain-of-function mechanism (Hagerman & Hagerman 2004, Oostra & Willemsen 2003).

FXTAS is a late-onset neurodegenerative disease involving gait instability, intention tremor, cognitive decline, and white matter abnormalities (Hagerman et al. 2001, Hagerman & Hagerman 2004). Autopsy studies have shown that the disease is characterized by eosinophilic ubiquitin-positive intranuclear astroglial inclusions and cerebellar degeneration (Hagerman & Hagerman 2004). Consistent with an RNA gain-of-function model, CGG-containing transcripts are found in the inclusions (Tassone et al. 2004). More recently, fluorescence-activated flow sorting has been used to isolate and perform mass spectrometry analysis on the inclusions, with the identification of a number of neurofilaments, lamin A/C, and two RNA-binding proteins, heterogeneous nuclear ribonucleoprotein A2 (hnRNP-A2) and MBNL1 (Iwahashi et al. 2005), which suggests possible downstream molecular parallels to DM.

Further evidence for an RNA gain-of-function mechanism comes from a *Drosophila* model in which a CGG repeat in the premutation size range was expressed in the 5' UTR of the *FMRI* gene (Jin et al. 2003). Flies developed neurodegeneration of the eye and ubiquitin-positive inclusions, providing further evidence that CGG premutation expansion transcripts are toxic. Additional support for an RNA gain-of-function mechanism comes from a mouse model in which a premutation repeat track (~100 CGGs) was introduced into the mouse *Fmr1* gene (Willemsen et al. 2003). Similar to humans, these mice

show cognitive and behavioral impairment (Van Dam et al. 2005). Additionally, these animals develop ubiquitin-positive intranuclear inclusions in the absence of increased FMR protein. These results strongly suggest that either the CGG expansion itself or elevated levels of FMR1 mRNA cause inclusion formation (Willemssen et al. 2003).

In summary, data from FXTAS patients as well as murine and fly models suggest that expression of CGG premutation transcripts cause the neurodegenerative changes associated with FXTAS and the formation of intranuclear inclusions (Hagerman & Hagerman 2004, Jin et al. 2003, Oostra & Willemssen 2003). A fascinating parallel between FXTAS and DM1 and DM2 is the recent discovery that MBNL1 is found in the inclusions (Iwahashi et al. 2005). This observation raises the possibility that dysregulation of MBNL1 or other proteins found in the inclusions may contribute to the CNS features of FXTAS, as well as those found in DM1 and DM2. Although FXTAS appears to be caused by an RNA gain-of-function mechanism, further research is required to determine if molecular changes such as the splicing alterations characteristic of DM are also found in FXTAS and if hnRNP-A1 and MBNL1 play a role in the disease.

### **Noncoding Expansions in the Spinocerebellar Ataxias**

**Spinocerebellar ataxia type 8.** In recent years, researchers have discovered that several of the dominant spinocerebellar ataxias are caused by mutations involving noncoding repeat expansions (**Figure 1b**). The first of these mutations was isolated from the DNA of a single patient using the repeat analysis pooled isolation and detection (RAPID) cloning (Koob et al. 1998, 1999). Similar to DM1, SCA8 provided a second example of a disease involving the expression of CUG expansion transcripts. In contrast to the multisystemic presentation of DM, SCA8 is a slow, progressive form of cerebellar ataxia,

characterized by gait and limb ataxia, nystagmus, and dysarthria (Day et al. 2000, Koob et al. 1999). Although repeat length and disease penetrance are tightly correlated in the initially described seven-generation MN-A kindred, the inheritance pattern is often complex, with only a subset of expansion carriers developing the disease (Cellini et al. 2001, Day et al. 2000, Ikeda et al. 2000, Koob et al. 1999, Topisirovic et al. 2002). Although the molecular basis for the reduced penetrance is not yet understood, factors that may influence whether a person with an SCA8 expansion will develop disease could include variations in the size of the CTA tract preceding the CTG expansion, possible expression differences among SCA8 patients and asymptomatic expansion carriers (Koob et al. 1999, Moseley et al. 2000, Stevanin et al. 2000), and sequence interruptions sometimes found within the repeat expansion itself (Martins et al. 2005).

SCA8 BAC transgenic mice expressing the expansion mutation develop a progressive neurological phenotype, demonstrating that expression of the human gene with the expansion, but not the control repeat tract, is pathogenic (Moseley et al. 2002). In a *Drosophila* model, retinal expression of SCA8 transcripts with both normal or expanded repeat tracts cause a neurodegenerative eye phenotype (Mutsuddi et al. 2004) with several genes, including muscleblind, split ends, staufer, and CG3249 identified as potential genetic modifiers of this phenotype (Mutsuddi et al. 2004). The fact that both the normal and expanded repeat tracts have similar effects in this fly model suggests that the cumulative effects of the overexpression of even very small CUG repeat tracts can trigger phenotypic effects. Similar to DM1, the genetic interaction between SCA8 CUG transcripts and muscleblind varies with repeat length (Mutsuddi et al. 2004).

**Spinocerebellar ataxia type 10.** The dominant noncoding mutation that causes SCA10 is a pentanucleotide ATTCT repeat

expansion within intron 9 of the *E46L* gene encoding the ataxin 10 protein (ATX10) (Lin & Ashizawa 2005, Marz et al. 2004) (**Figure 1b**). The SCA10 expansion mutation is one of the largest, with sizes ranging from 800 to 4500 repeats. The typical clinical presentation of SCA10 involves cerebellar dysfunction and seizures with variable expression of polyneuropathy, pyramidal signs, as well as cognitive and neuropsychiatric impairment (Lin & Ashizawa 2005, Rasmussen et al. 2001).

ATX10 is an evolutionarily conserved cytoplasmic protein (475 amino acids) of unknown function. The *E46L* gene is broadly expressed, with significant expression in the brain, testis, kidney, heart, and skeletal muscle (Marz et al. 2004, Matsuura et al. 2000). Preliminary studies with yeast two-hybrid analysis identified a subunit of heterotrimeric GTP-binding protein (G protein) as a potential interacting protein, suggesting that ATXN10 may promote G-protein signaling, which leads to increased neurite formation (Lin & Ashizawa 2005). A separate study showed that knock down of SCA10 transcripts in cultured cerebellar and cortical neurons caused increased apoptosis, with cerebellar neurons significantly more sensitive to reduced SCA10 expression levels (Marz et al. 2004). Although these data suggest the possibility that loss of protein could affect neurite outgrowth or mediate other neurotoxic effects, initial studies showing that even very large ATTCT repeat tracts do not reduce transcript levels suggest that expression of E46L transcripts may in fact not be reduced in patients and may not be a clinically relevant model. Clearly, additional studies are needed to determine the effects of the ATTCT expansion on expression of ataxin 10 in patients (Bidichandani et al. 1998, Matsuura et al. 2000).

Interestingly, overexpression of a pure ATTCT tract in cell culture can cause the accumulation of AUUCU-containing RNA and foci formation (Lin & Ashizawa 2005, Marz et al. 2004, Matsuura et al. 2000). Ad-

ditional experiments are needed to determine if similar inclusions are found in human tissue. It is also important to determine whether Mbn11, CUG-BP1, or other proteins bind to the AUUCU expansions and if downstream molecular effects on splicing of other genes in *trans* are also observed.

**Spinocerebellar ataxia type 12.** A third dominant spinocerebellar ataxia (SCA12) is caused by a noncoding CAG expansion mutation at the 5' end of the *PPP2R2B* gene (**Figure 1b**). Patients with SCA12 are variably affected by action tremor, cerebellar dysfunction, hyperreflexia, subtle Parkinsonian features, and dementia (Holmes et al. 2003, O'Hearn et al. 2001). The *PPP2R2B* gene encodes a brain-specific regulatory subunit of the protein phosphatase PP2A holoenzyme. Depending on the transcription initiation site, the CAG repeat tract can be located in the 5' UTR or the upstream promoter region of the gene with no evidence to date that the repeat is located within an open reading frame or that it encodes a protein. Studies examining the effects of the repeat tract on the expression of a reporter demonstrated that the expanded repeat can cause an increase in expression (Sowell et al. 2000). On the basis of these data, researchers have suggested that the CAG expansion may cause increased *PPP2R2B* expression, which in turn may alter PP2A activity, resulting in changes in protein phosphorylation. Because some forms of the transcripts contain the CAG repeat, a second model involving a gain-of-function RNA mechanism is also possible (Holmes et al. 1999, 2001a, 2003).

### Huntington's Disease-Like 2

HDL2, a disease with a number of clinical parallels to Huntington's disease, is a dominant disorder characterized by movement abnormalities, dementia, and psychiatric problems (Holmes et al. 2001b). Neuropathology shows cortical and striatal atrophy and, interestingly, the presence of intranuclear inclusions



(Margolis et al. 2005). HDL2 is caused by a CTG expansion in the junctophilin-3 gene (*JP-3*), with locations of the repeat expansion mutation in the coding or intronic regions or as part of the 3' UTR, depending on the alternative splicing pattern (Figure 1*b*). Potentially pathogenic poly-leucine or poly-alanine tracts are predicted to result from two known splice forms, and the expression of CUG expansion transcripts may trigger gene dysregulation, resulting in a toxic RNA effect (Holmes et al. 2001*b*). Although it is not yet clear what lessons will be learned from understanding how the HDL2 expansion mutation causes disease, understanding the molecular mechanism of HDL2 likely holds important lessons for understanding the pathogenesis of both Huntington's and other neurodegenerative diseases.

## CONCLUSIONS

The realization of the prominent role for toxic RNA in DM1 pathogenesis came about only as more conventional models fell by the wayside. Dominant inheritance of DM1 was not explained by haploinsufficiency of *DMPK* or flanking genes, and the discovery of the DM2 mutation provided compelling support for the hypothesis that the unusual group of multisystemic features common to both diseases result from a pathogenic mechanism involving the dysregulation of RNA-binding proteins, mediated by the expression of CUG and CCUG expansion transcripts (Liquori et al. 2001). SCA8, SCA10, SCA12, HDL2, and FXTAS provide additional examples of what appear to be a growing group of dominant diseases caused by noncoding expansions. Although an RNA gain-of-function mechanism may play a role in the grow-

ing number of known noncoding expansion disorders, it is interesting that mutations at some of these loci can have dramatically different clinical consequences, which in some cases has slowed the progress in recognizing the diverse molecular consequences of these mutations. For instance, CGG full-mutation expansions at the *FMRI* locus cause severe mental retardation with an early age of onset through a loss-of-function mechanism in which no FMRP protein is expressed, whereas premutation expansions cause a completely different late-onset tremor ataxia syndrome likely triggered by a gain-of-function RNA mechanism involving the expression of CGG expansion transcripts. Other examples of variable phenotypes caused by mutations at single genetic loci include (*a*) moderate CTG expansion sizes in DM1, which sometimes cause a relatively mild adult-onset disease but can also result in severe congenital-onset DM1 (Redman et al. 1993, Steinbach et al. 1998) and (*b*) the SCA8 CTG expansion mutation, which can cause a progressive neurodegenerative spinocerebellar ataxia. However, SCA8 expansions, which can be found in the general population, often do not cause disease. The molecular basis for these notable variations in disease presentation and reduced penetrance will become clearer as the molecular mechanisms of noncoding expansion disorders are more fully understood. With the complexities of these diseases in mind, it may be instructive to carefully consider what underlying molecular pathways are being ignored in other diseases because our bias as scientists has been to focus first on the most apparent molecular alterations, which, until quite recently, have been almost exclusively considered the effects that mutations have on the corresponding protein encoded by the same gene.

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