Misregulation of Alternative Splicing Causes Pathogenesis in Myotonic Dystrophy

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Abstract. Myotonic dystrophy (DM), the most common form of adult onset muscular dystrophy, affects skeletal muscle, heart, and the central nervous system (CNS). Mortality results primarily from muscle wasting and cardiac arrhythmias. There are two forms of the disease: DM1 and DM2. DM1, which constitutes 98% of cases, is caused by a CTG expansion in the 3′ untranslated region (UTR) of the *DMPK* gene. DM2 is caused by a CCTG expansion in the first intron of the *ZNF9* gene. RNA containing CUG- or CCUG-expanded repeats are transcribed but are retained in the nucleus in foci. Disease pathogenesis results primarily from a gain of function of the expanded RNAs, which alter developmentally regulated alternative splicing as well as pathways of muscle differentiation. The toxic RNA has been implicated in sequestration of splicing regulators and transcription factors thereby causing specific symptoms of the disease. Here we review the proposed mechanisms for the toxic effects of the expanded repeats and discuss the molecular mechanisms of splicing misregulation and disease pathogenesis.

1 Myotonic Dystrophy

DM is a multisystemic, autosomal dominant disorder that is the most common form of adult onset muscular dystrophy. Manifestations of the disease are highly variable, consisting of muscular, neuronal, and endocrine features, each of which may vary in severity. Muscle dysfunction is the most common symptom including muscle weakness, pain, and myotonia (difficulty relaxing muscle after voluntary contraction). Cardiac symptoms include conduction defects and arrhythmias, potentially resulting in sudden death. Endocrine abnormalities result in glucose intolerance. Testicular failure is common and is associated with sterility (Harper 2001).

Myotonic dystrophy type I (DM1) is the most common form, accounting for approximately 98% of DM cases. DM1 is caused by a CTG expansion in the 3′UTR of the myotonic dystrophy protein kinase (*DMPK*) gene on chromosome 19q13.3 (Brook et al. 1992; Fu et al. 1992; Mahadevan et al. 1992). While *DMPK* alleles in unaffected individuals contain 5–34 repeats, expanded alleles can reach 50–2000 repeats in individuals with

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DM1. The prevalence of DM1 is estimated to be 1 in 8,000 worldwide (Harper 2001).

Longer repeat lengths correlate with a younger age of onset and increased severity of the disease (Tsilfidis et al. 1992). The most severe form of DM1 is congenital myotonic dystrophy (CDM) in which repeat sizes are >1,400. CDM is characterized by neonatal hypotonia, respiratory failure, which is often fatal, facial diplegia, and mental retardation (Harper 2001). The hypotonia in infants with CDM is thought to involve a developmental defect in skeletal muscle differentiation and maturation. The survival rate for individuals with CDM is approximately 50%. Those that survive improve but then later develop adult DM symptoms in their second or third decade (Harper 2001). These observations suggest that CDM and adult onset disease significantly differ in that CDM represents a developmental abnormality while adult onset disease represents a degenerative process.

DM type 2 (DM2), also called proximal myotonic myopathy (PROMM), makes up 2–3% of myotonic dystrophy cases (Harper 2001). The mutation that causes DM2 is a CCTG expansion in the first intron of the *ZNF9* gene located on chromosome 3q21 (Liquori et al. 2001). DM2 appears to be a milder disease than DM1 but DM2 expansions are larger ranging from 75 to 11,000 CCTG repeats. The clinical presentation of DM1 and DM2 are strikingly similar, however, there are important differences, potentially reflecting mechanistic differences in disease pathology. A congenital form of DM2 has not been identified. In addition, DM1 is associated with atrophy of type 1 skeletal muscle fibers in contrast to DM2, in which atrophy of type 2 fibers is observed (Tohgi et al. 1994).

Recently, a novel multisystemic myotonic disorder has been identified in a large French pedigree, associated with frontotemporal dementia (Le Ber et al. 2004). Histological features in muscle and brain were similar to histological features of DM and the causative region was mapped within chromosome 15 (15q21–24). Therefore, the disease was designated as DM type 3 (DM3). The causative mutation has not been identified.

2
Repeat Instability

DM is typified by anticipation in which disease severity increases in successive generations. The molecular basis for anticipation is germline instability in which repeats expand. In addition to germ line instability, the repeat sizes in DM1 alleles expand in somatic tissues during aging (Ashizawa et al. 1993; Martorell et al. 2004; Wong and Ashizawa 1997). The molecular mechanisms for the genetic instability of triplet repeat expansions have been extensively investigated in bacteria, yeast, and mice (Cummings and Zoghbi 2000; Gomes-Pereira et al. 2004;

Gourdon et al. 1997; Pearson et al. 1997; Savouret et al. 2003; Wells 1996). One proposed mechanisms for genetic instability is the DNA polymerase slippage model, which predicts that repeat size variability arises during DNA replication in a cell-division dependent manner (Richards and Sutherland 1994). Folding of expanded CTG repeats into hairpin or alternative non-B DNA structures is thought to cause slippage of DNA polymerase during replication due to mismatched base pairs (Gacy et al. 1995; Gellibolian R 1997). In support to this, NMR studies showed that CTG repeats can form three different types of hairpin structures generating mismatched base pairs, which allows expansion of repeats during DNA replication (Chi and Lam 2005).

Transgenic mouse models have been used to establish a strong link between mismatch repair (MMR) proteins and repeat instability. For example, transgenic mice containing the entire human *DMPK* gene with 55-CTG repeats obtained from a mildly affected patient showed both intergenerational and somatic repeat instability as observed in individuals with DM (Gourdon et al. 1997). When transgenic mice expressing expanded CTG repeats in the mouse *Dmpk* gene were mated with mice lacking individual MMR proteins, the instability of expanded CTG repeats was altered. In mice lacking *Msh3*, the instability of CTG repeats was corrected. Conversely, the instability of repeats worsened in mice lacking *Msh6* (Pearson et al. 1997). The absence of *Msh2* in transgenic mice expressing >300 unstable CTG repeats favored contractions of the repeats, both in tissues and through generations (Savouret et al. 2003). When transgenic mice deficient for *Pms 2* were mated with mice expressing CAG/CTG repeats, the rate of somatic expansions were reduced by 50% and a higher frequency of large deletions was detected (Gomes-Pereira et al. 2004). On the other hand, deletion of genes involved in various DNA repair pathways like *Rad52*, *Rad54* (homologous recombination) and *DNA-PKc*s (non-homologous end-joining) did not affect repeat instability (Savouret et al. 2003). These results indicate that MMR proteins are involved in different aspects of triplet repeat instability. In addition to the MMR pathway, methylation is found to be important for repeat instability. Expansions of CTG repeats were destabilized in DM1 cells in the presence of DNA methyltransferase inhibitors (Gorbunova et al. 2004). These results indicate that there is more than one mechanism involved in CAG/CTG repeat instability.

3
Mechanism of DM Pathogenesis

The mutant alleles containing CTG or CCTG expansions are transcribed and processed normally into polyadenylated and spliced mRNAs. The mature *DMPK* mRNAs containing the expanded CUG repeats are not

exported to the cytoplasm but rather accumulate in nuclear foci detectable by in situ hybridization (Davis et al. 1997; Fardaei et al. 2001; Fardaei et al. 2002; Taneja et al. 1995). Similarly, the excised intron from the expanded *ZNF9* allele also accumulates in nuclear foci (Liquori et al. 2001; Ranum and Day 2002). However, it is unclear how the CTG and CCTG expansions in noncoding regions cause a multisystemic disease.

Three hypotheses have been proposed for the molecular mechanisms of DM pathogenesis: (1) loss of function of *DMPK*, (2) loss of function of surrounding genes, and (3) RNA gain of function. Knockout mouse models generated to test the loss of function of DMPK and surrounding genes were only mildly related to a DM phenotype. In contrast, the transgenic animal models with expanded CTG repeats strongly supported the RNA "gain of function" hypothesis.

3.1 Loss of Function of DMPK

DMPK is a serine–threonine kinase expressed in skeletal muscle, heart, and to a lesser extent in brain and testes (Lam et al. 2000; Ueda et al. 2000). *DMPK* transcripts are subject to cell-type-dependent alternative splicing (Groenen et al. 2000; Wansink et al. 2003). All isoforms contain CTG repeats in the 3′ UTR except one isoform that splices out the repeats using an alternative splice acceptor site in exon 15 (Tiscornia and Mahadevan 2000). The biological function of *DMPK* is unknown, however, data suggest that DMPK protein might be involved in regulation of actin cytoskeleton (Jin et al. 2000) and in calcium homeostasis (Kaliman et al. 2005). In addition, specific *DMPK* splice variants localize to endoplasmic reticulum and mitochondrial membranes, and their presence causes ER and mitochondrial clustering (van Herpen et al. 2005). In individuals with DM1, nuclear retention of *DMPK* mRNA from the expanded allele results in reduced DMPK protein levels (Ueda et al. 1999). Thus, it was proposed that low levels of DMPK protein contribute to disease pathogenesis. To understand the function of *DMPK* and its role in DM pathogenesis, *Dmpk* knockout mice were generated (Reddy et al. 1996). These mice developed cardiac conduction abnormalities (Berul et al. 1999; Reddy et al. 1996; Saba et al. 1999), altered calcium homeostasis (Benders et al. 1997), abnormal sodium channel gating (Mounsey et al. 2000), and reduced skeletal muscle force (Reddy et al. 1996), suggesting that *Dmpk* may be involved in maintenance of muscle fiber. While a *Dmpk* knockout mouse displayed the relatively mild symptoms observed in DM1 patients, it did not reproduce the most characteristic and severe features of the disease like myotonia or muscle wasting.

Transgenic mice overexpressing *DMPK* developed hypertrophic cardiomyopathy and increased neonatal mortality (Jansen et al. 1996), however these transgenic mice did not show prominent features of DM.

3.2 Loss of Function of Surrounding Genes

Expanded CTG repeats were shown to alter chromatin structure and have regional effects on gene expression (Otten and Tapscott 1995; Wang et al. 1994). The CTG expansion in the *DMPK* 3′UTR are located immediately upstream of the *SIX5* promoter region and were shown to lower *SIX5* expression (Gennarelli et al. 1999; Inukai et al. 2000; Klesert et al. 1997; Thornton et al. 1997). Six5 is a transcription factor required for eye development in *Drosophila*, and the mouse homologue is implicated in distal limb muscle development (Harris et al. 2000). *Six5* knockout mice develop ocular cataracts and infertility resembling some features of DM1 (Klesert et al. 2000; Sarkar et al. 2000). Cardiac conduction abnormalities were also noted in *Six5* knockout mice (Wakimoto et al. 2002). However, the most common symptoms of DM1 such as muscle weakness, wasting, and myotonia were not reproduced in *Six5* knockout mice (Klesert et al. 2000; Sarkar et al. 2000). The identification of a second locus causing DM2 reduced the likelihood that loss of function of *DMPK* or flanking genes was the determinative mechanism for at least the symptoms common for DM1 and DM2.

3.3 RNA "Gain of Function" Hypothesis

Transgenic mice expressing 250 CTG in the final exon of the human skeletal alpha actin gene (HSA_{250}) displayed characteristics of the DM phenotype (Mankodi et al. 2000). Specifically, HSA_{250} mice but not mice expressing transgenes containing five repeats (HSA_5) developed myotonia, a classical feature of DM (Mankodi et al. 2000). Muscle histology showed increased central nuclei, ringed fibers in muscle, and variability in fiber size similar to histological features observed in individuals with DM1. Nuclear foci were detected by in situ hybridization. These mice had a higher mortality rate than normal controls. The reason for increased mortality was unclear (Mankodi et al. 2000). These results suggested that CTG repeats in the absence of *DMPK* mRNA are sufficient to cause several DM symptoms and strongly supported a hypothesis proposing an RNA gain of function (Timchenko et al. 1996a; Wang et al. 1995). On the other hand, HSA_{250} mice did not develop muscle weakness or wasting, indicating that there are likely to be other determinants involved in disease pathogenesis.

A second set of transgenic mice expressing 300 CUG repeats in the natural context of the human *DMPK* mRNA developed mild muscle and brain abnormalities consistent with DM1 (Seznec et al. 2001). Histological abnormalities in muscle included central nuclei, mild muscle regeneration, degeneration, and altered mitochondrial morphology without significant muscle

weakness and wasting. The authors detected myotonia in transgenic mice by EMG (Seznec et al. 2001). Unlike HSA_{250}, expression of RNA containing expanded CUG repeats was not limited to skeletal muscle. These transgenic mice showed abnormal tau protein expression in the brain similar to DM1 patients, providing evidence for toxic effects of CUG expansion in CNS.

Here we summarize the additional evidence for an RNA gain-of-function hypothesis. First, the fact that two different loci containing similar expanded repeats cause strikingly similar diseases strongly suggest that DM1 and DM2 pathogenesis is independent of a loss of function of the affected loci. Second, only the repeats and no other mutations within the DM1 or DM2 locus cause DM, indicating that the expanded repeats themselves rather than a loss of function of the mutant alleles are determinative for the disease. Third, the RNA transcribed from the mutated allele containing expanded repeats (CUG/CCUG) accumulates in discrete nuclear foci detectable by in situ hybridization (Liquori et al. 2001; Taneja et al. 1995). Fourth, *Dmpk* and *Six5* knockout mice do not reproduce a strong DM phenotype (Benders et al. 1997; Berul et al. 1999; Klesert et al. 2000; Reddy et al. 1996; Saba et al. 1999; Sarkar et al. 2000). These results indicate that expression of expanded CUG or CCUG repeats independent of the loci is sufficient to induce the major features of the disease.

The expression of CUG or CCUG repeat containing RNAs is proposed to induce pathogenesis by at least three mechanisms: (1) misregulation of pre-mRNA alternative splicing, (2) interference with muscle differentiation, and (3) transcriptional interference. Each of these potential mechanisms will be discussed below.

3.3.1 Misregulation of Alternative Splicing

Alternative splicing is a process by which multiple mRNA isoforms are generated from individual genes. The majority of human genes undergo alternative splicing explaining, in part, the disparity between the relatively small number of genes and the complexity of the human proteome (Modrek and Lee 2002; Xu et al. 2002). Alternative splicing gives rise to protein isoforms that significantly differ in their functions (Black 2003). Alternative splicing is often regulated according to cell type or developmental stage. Regulation involves binding of regulatory factors to intronic or exonic elements (Black 2003). The regulation of alternative splicing can have an enormous impact on multiple aspects of cell and tissue physiology (Lopez 1998). Aberrant regulation of alternative splicing has been implicated in several human diseases (Faustino and Cooper 2003; Lopez 1998). Ten misregulated alternative splicing events that have been identified in DM1 heart, skeletal muscle, and central nervous system are summarized in Table 1.

IR splicing has been shown to be misregulated in DM2 skeletal muscle consistent with a similar pathogenic mechanism as in DM1 (Savkur et al.

Table 1. Summary of alternative splicing events misregulated in DM1

Pre-mRNA	Mis-regulated exon/intron	Reference
Cardiac troponin T (*TNNT2* or *cTNT*)	exon 5	Philips et al. (1998)
Insulin receptor (*IR*)	exon 11	Savkur et al. (2001)
Chloride channel (*CLCN-1*)	intron 2 and exon 7a	Charlet-B. et al. (2002b); Mankodi et al. (2002)
Microtubule-associated protein tau (*MAPT*)	exon 2 and 10	Sergeant et al. (2001); Jiang et al. (2004)
Myotubularin-related protein 1 *MTMR1*	exons 2.1 and 2.3	Buj-Bello et al. (2002)
Fast skeletal troponin T (*TNNT3*)	fetal exon	Kanadia et al. (2003a)
N-methyl-D-aspartate receptor (*NMDAR1*)	exon 5	Jiang et al. (2004)
Amyloid precursor protein (*APP*)	exon 7	Jiang et al. (2004)

2004). In all cases, the regulation of alternative splicing is disrupted such that normal mRNA variants are expressed, but in inappropriate tissues or developmental stages. Alternative splicing of only a subset of genes is misregulated in DM indicating that most genes are unaffected (Jiang et al. 2004; Philips et al. 1998). Interestingly, all pre-mRNAs misregulated in DM1 normally undergo a developmentally regulated splicing switch. In DM adult tissues, the embryonic or fetal splicing patterns for these genes are retained. Misexpression of the early developmental isoforms for *IR* and *CLCN-1* has been shown to directly correlate with disease symptoms such as insulin resistance and myotonia, respectively (Charlet-B. et al. 2002b; Mankodi et al. 2002; Savkur et al. 2001). The next section summarizes all the pre-mRNAs that are misspliced in individuals with DM.

3.3.1.1 TNNT2 (*cTNT*) Contraction of striated muscle is regulated by binding of calcium to the troponin complex located on the actin-based thin filament. This complex consists of troponin T, troponin I, and troponin C (TNT, TNI, and TNC, respectively). This complex regulates the calcium-dependent interaction of actin and myosin that results in muscle contraction (Cullen et al. 2004).

TNNT2 is the cardiac isoform of TNT, which is the gene expressed in embryonic heart, embryonic skeletal muscle, and adult cardiac muscle

(Anderson et al. 1991). Alternative splicing of exon 5 is regulated such that the exon is included in mRNAs produced during early development of heart and skeletal muscle but the exon is skipped in adult heart (Anderson et al. 1995). The two major TNNT2 isoforms generated by alternative splicing of exon 5 confer different calcium sensitivity to the myofilament, affecting the contractile properties of maturing muscle (Godt et al. 1993; McAuliffe et al. 1990). *TNNT2* alternative splicing is disrupted in DM1 such that exon 5 is inappropriately included in adult cardiac muscle (Philips et al. 1998). Mutations in *TNNT2* and *cTNI* genes are associated with inherited heart diseases including hypertrophic and dilated cardiomyopathies (Lu et al. 2003). Specifically, mutations in *TNNT2* gene are implicated in dominantly inherited familial cardiomyopathies (Forissier et al. 1996; Nakajima-Taniguchi et al. 1997; Thierfelder et al. 1994; Watkins et al. 1995). Thus, the expression of fetal *TNNT2* isoform in DM1 patients might contribute to the reduced myocardial function and conduction abnormalities seen in DM patients.

3.3.1.2 TNNT3 The *TNNT3* gene encodes the TNT isoform expressed in fast-twitch skeletal muscle myofibers. A fetal exon is located between exons 8 and 9 of *TNNT3* gene and this fetal exon is inappropriately included in adult DM1 skeletal muscle (Kanadia et al. 2003a). The functional consequences of the inappropriate isoform are unknown.

3.3.1.3 IR IR is a tetrameric complex with two alpha and two beta subunits. Binding of insulin to the extracellular alpha subunits causes autophoshorylation of intracellular beta subunits (Joost 1995; Kellerer et al. 1992). Alternative splicing of exon 11 of the alpha subunit generates two isoforms: IR-A, which lacks exon 11, and IR-B, which includes exon 11 (Mosthaf et al. 1990; Seino and Bell 1989). Expression of the two isoforms is regulated in a tissue-specific manner such that IR-B is expressed predominantly in tissues responsible for glucose homeostasis such as liver, adipose tissue, and skeletal muscle (Condorelli et al. 1994). IR-A has a higher affinity for insulin with lower signaling capacity and is expressed at low levels in these tissues (Kosaki et al. 1995; Vogt et al. 1991). The inappropriate expression of IR-A in skeletal muscle directly correlates with the insulin resistance seen in DM1 and DM2 patients (Savkur et al. 2001; Savkur et al. 2004).

3.3.1.4 ClC-1 The muscle-specific chloride channel (ClC-1), encoded by *CLCN-1* gene, is the predominant chloride channel in adult skeletal muscle (Bardouille et al. 1996; Pusch 2002), and loss of function mutations in this gene results in inherited myotonias in humans and other mammals (Beck et al. 1996; Koch et al. 1992; Rhodes et al. 1999; Zhang et al. 2000). Aberrant splicing of the *CLCN-1* pre-mRNA results in the loss of CIC-1 protein in skeletal muscle of individuals with DM1 or

DM2 due to introduction of premature termination codons, which is thought to trigger nonsense mediated decay, resulting in degradation of *CLCN-1* mRNA (Charlet-B. et al. 2002b; Mankodi et al. 2002). The loss of CIC-1 correlates well with the myotonia observed in individuals with DM1 and DM2.

3.3.1.5 Tau Tau (encoded by the *MAPT* gene) is a microtubule-associated protein that is required for polymerization and stability of microtubules involved in axonal transport (Buee et al. 2000; Goedert et al. 1992). Exons 2, 3 and 10 are alternative exons that are developmentally regulated giving rise to six different isoforms (Andreadis et al. 1992). Exon 10 encodes an additional microtubule-binding domain, which increases its affinity to microtubules (Hartmann et al. 2001; Varani et al. 2000). Exon 2 alters the structure and function of the membrane-binding domain of tau allowing connection of microtubules to the axonal membrane (Brandt et al. 1995; Li et al. 2003). Exon 10 is not included in fetal brain but is included in 50% of the transcripts in adult brain. Similar to exon 10, exons 2 and 3 are excluded in fetus but included in adult. Two independent studies demonstrated that fetal forms of *MAPT* (excluding exons 2, 3 and 10) were inappropriately expressed in adult brain of individuals with DM1 (Jiang et al. 2004; Sergeant et al. 2001). Expression of human fetal tau isoforms in transgenic mice results in neurofibrillary tangles which are also seen in other neurological diseases (Andreadis 2005; Gotz et al. 2001; Ishihara et al. 2001). In addition, neurofibrillary tangles can be detected in brains of individuals with DM1 (Kiuchi et al. 1991; Vermersch et al. 1996). These observations raise the possibility that expression of a fetal tau isoform might be involved in production of neurofibrillary tangles affecting behavioral and cognitive functions in individuals with DM1.

3.3.1.6 APP The characteristic features of Alzheimer's disease are senile plaques and neurofibrillary tangles in the brains of affected individuals. The major component of senile plaques is amyloid, a peptide derived from proteolysis of a large beta-amyloid precursor protein (APP). APP is a type I trans-membrane glycoprotein existing in eight isoforms generated by alternative splicing of exons 7, 8, and 15 (Sandbrink et al. 1996). Exon 7 is believed to encode a serine protease inhibitor domain (Ponte et al. 1988). The *APP* mRNA, which excludes exon 2 and 7 is fetus-specific (Tang et al. 2003). Fetal forms of *APP* excluding exon 7 are inappropriately expressed in brains of individuals with DM (Jiang et al. 2004). The consequences for the loss of this protease inhibitory domain by exclusion of exon 7 in DM1 are unclear.

3.3.1.7 NMDAR1 N-methyl-D-aspartate receptors (NMDAR) are involved in excitatory transmission in the mammalian brain and are crucial for brain development, learning, and memory (Sato et al. 2000; Tsien et al.

1996). *NMDAR1* pre-mRNA has three alternative exons: 5, 21, and 22. Differential usage of these exons generates at least seven mRNA isoforms that encode proteins with different physiological properties and subcellular distribution (Durand et al. 1993; Zukin and Bennett 1995). The protein segment encoded by exon 21 is believed to be important for localization of NMDAR1 to the post-synaptic plasma membrane (Ehlers et al. 1995). The *NMDAR1* isoform including exon 21 was increased in brain tissues of individuals with DM1 (Jiang et al. 2004). Similarly, the inclusion of exon 5 of *NMDAR1* is thought to affect the intracellular distribution of NMDAR1 (Pal et al. 2003; Traynelis et al. 1995). Increased exon 5 and exon 21 inclusion was detected in brain tissues of DM1 patients (Jiang et al. 2004), implicating a possible correlation between a change in the distribution of NMDAR1 and the CNS symptoms of DM.

3.3.1.8 MTMR1 The *MTMR1* gene belongs to a conserved family of phosphatidylinositol 3-phosphate [PI (3)P] phosphatases (Laporte et al. 2001) involved in regulation of intracellular vesicular trafficking and membrane transport (Simonsen et al. 2001). Alternative splicing of exons 2.1, 2.2, and 2.3 generates the muscle-specific protein isoforms A, B, and C (Buj-Bello et al. 2002). Exons 2.1, 2.2, and 2.3 encode 8, 9, and 17 amino acids, respectively (Buj-Bello et al. 2002). A switch from isoform A to C is detected in individuals with CDM. It is unclear whether there is a change in protein function due to the isoform switch since phosphatase activities seems to be similar (Buj-Bello et al. 2002). However, there are two independent reports suggesting that point mutations or deletions in the *MTMR1* gene are associated with myotubular myopathy, a disease associated with hypotonia and respiratory insufficiency resembling some features of CDM (Copley et al. 2002; Zanoteli et al. 2005). In addition, the loss of *MTM1*, a gene closely related to *MTMR1*, is implicated in a congenital muscular disorder called X–linked myotubular myopathy, exhibiting some similarities to CDM such as hypotonia, muscle weakness, and muscle fibers with central nuclei (Wallgren-Pettersson et al. 1995).

3.4 Mechanisms of Misregulated Alternative Splicing

The specific mechanism by which expression of CUG- or CCUG-repeat RNA induces splicing misregulation is unclear. However, there is substantial evidence linking the misregulation of alternative splicing observed in DM tissues with two families of RNA binding proteins: CUG-BP and ETR-3-Like Factors (CELF) and muscleblind-like (MBNL). Members of both the CELF and MBNL families were first identified based on their binding to CUG-repeat RNA in vitro (Lu et al. 1999; Michalowski et al. 1999; Miller et al. 2000; Timchenko et al. 1996b). Members of both protein

families have been demonstrated to bind RNA and to directly regulate alternative splicing of multiple pre-mRNAs including several that undergo misregulated alternative splicing in DM (Charlet-B. et al. 2002b; Ho et al. 2004; Philips et al. 1998; Savkur et al. 2001). Interestingly, CELF and MBNL proteins have been shown to antagonistically regulate two splicing events that are misregulated in DM tissues (*TNNT2* and *IR*), and the splicing patterns of at least these two pre-mRNAs are consistent with either loss of MBNL activity and/or a gain of CELF activity (Ho et al. 2004). There is evidence strongly supporting both increased CELF activity and reduced MBNL activity as determinative factors in misregulated splicing in DM. Each of these families will be described as well as their potential role in misregulated alternative splicing.

3.4.1 Increased CUG-BP1 Splicing Activity

There are six CELF paralogues in humans: ETR-3 (CUG-BP2/BRUNOL3/NAPOR), CELF3 (BRUNOL1), CELF4 (BRUNOL4), CELF5 (BRUNOL5), CELF6 (BRUNOL6), and CUG-BP1 (BRUNOL2/CUG-BP1). The CELF paralogues are 43–78% identical and all six have three RNA recognition motifs (RRMs) and a 160–230 amino acid divergent domain separated by RRMs 2 and 3 (Ladd et al. 2001). CELF proteins are involved in both nuclear and cytoplasmic events such as alternative splicing, RNA editing, and mRNA stability and translation (Anant et al. 2001; Ladd et al. 2001; Ladd et al. 2004; Mukhopadhyay et al. 2003; Timchenko et al. 1999). With regard to alternative splicing, CELF proteins have been shown to regulate a number of pre-mRNAs by directly binding to U/G-rich motifs within introns (Charlet-B. et al. 2002a; Charlet-B. et al. 2002b; Faustino and Cooper 2005; Philips et al. 1998). Alternatively spliced genes that are regulated by CELF proteins include *TNNT2* exon 5, *IR* exon 11, *CLCN-1* intron 2, *NMDAR1* exons 5 and 21, actinin muscle-specific exon, and *MTMR1* exon 2.1 and 2.2 (Charlet-B. et al. 2002a; Charlet-B. et al. 2002b; Faustino and Cooper 2005; Gromak et al. 2003; Philips et al. 1998; Savkur et al. 2001; Zhang et al. 2002).

CUG-BP1 is the most studied member of the CELF protein family. CUG-BP1 has been demonstrated to directly regulate three alternative splicing events that are misregulated in DM: *CLCN-1* intron 2, *TNNT2* exon 5, and *IR* exon 11 (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). In tissue culture, the misregulated splicing patterns observed for these three genes in DM1 tissues can be recapitulated by overexpression of CUG-BP1, suggesting that DM cells exhibit an increased activity of CUG-BP1 or other members of the CELF family (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). Consistent with increased CUG-BP1 splicing activity, CUG-BP1 protein levels are increased in DM1 skeletal muscle tissue (Savkur et al. 2001), DM1 skeletal muscle cultures

(Dansithong et al. 2005; Savkur et al. 2001) and in DM1 heart tissue (Timchenko et al. 2001a). In addition, transgenic mice expressing CUG-BP1 eight to ten fold above endogenous levels inhibited muscle differentiation and resulted in neonatal lethality (Timchenko et al. 2004). Histological features were variably consistent with what is observed in CDM patients including centrally positioned nuclei suggestive of immature skeletal muscle. However, type 1 slow myofiber numbers were increased while these are decreased in DM skeletal muscle. The mechanism of muscle immaturity is thought to be due to altered translation of p21 and myogenin mRNAs by CUG-BP1. In another line of transgenic mice overexpressing CUG-BP1 in heart and skeletal muscle using the mouse creatine kinase promoter (MCKCUG-BP), transgene expression was associated with neonatal lethality (Ho et al. 2005a). Histological changes were consistent with CDM, as well as splicing changes observed for *TNNT2* exon 5 and *MTMR1* exons 2.1 and 2.2 in cardiac muscle, and *MTMR1* exons 2.1 and 2.2 and *CLCN-1* exon 7a in skeletal muscle tissue (Ho et al. 2005a).

3.4.2 Sequestration of MBNL Proteins

The three human MBNL paralogues are homologues of *Drosophila* muscleblind (*mbl*), which is required for *Drosophila* photoreceptor and muscle differentiation (Artero et al. 1998; Begemann et al. 1997). *MBNL1*, *MBNL2*, and *MBNL3* are located on chromosomes 3, 13, and X, respectively (Fardaei et al. 2002; Miller et al. 2000). MBNL1 was identified based on its ability to bind double-stranded CUG-repeat RNA in HeLa cell nuclear extracts (Miller et al. 2000). All three MBNL proteins colocalize with expanded CUG and CCUG RNA nuclear foci in cultured cells as detected by immunofluorescence (Fardaei et al. 2002; Mankodi et al. 2001; Miller et al. 2000). In addition, MBNL can bind to expanded (up to 50) CCUG, CUG, and CAG repeats as detected by a yeast three-hybrid assay (Kino et al. 2004). Both MBNL1 and MBNL2 are expressed in skeletal muscle and heart, two tissues that are prominently affected in DM (Kanadia et al. 2003b). MBNL3 expression is restricted to the placenta in the adult mice and is more widely expressed in the embryo (Fardaei et al. 2002; Miller et al. 2000).

The observations that muscleblind proteins colocalize with the expanded CUG and CCUG repeats strongly suggests that loss of MBNL function due to sequestration on CUG-repeat RNA plays a major role in DM pathogenesis (Dansithong et al. 2005; Fardaei et al. 2002; Jiang et al. 2004; Mankodi et al. 2001; Miller et al. 2000). Results from *Mbnl* knockout mice strongly support this hypothesis (Kanadia et al. 2003a). Targeted deletion of exon 3 in mice ($MBNL1^{\Delta E/\Delta E}$) to eliminate the MBNL1 isoforms that bind expanded CUG/CCUG RNA repeats resulted in myotonia, cataracts, and RNA splicing defects that are striking characteristics of DM.

Histological analysis of muscle revealed increased central nuclei and splitting of myofibers. MBNL1ΔE/ΔE mice showed abnormal retention of the *TNNT3* fetal exon and *CLCN-1* exon7a in skeletal muscle and *TNNT2* exon 5 in heart consistent with splicing changes seen in individuals with DM1 and without changes in steady state levels of CUG-BP1 (Kanadia et al. 2003a).

The MBNL family was recently identified as direct regulators of alternative splicing (Ho et al. 2004). Specifically, MBNL proteins regulate splicing of *TNNT2* exon 5 and *IR* exon 11 via direct binding to adjacent intronic elements (Ho et al. 2004). MBNL and CELF proteins have antagonistic effects on the splicing patterns of these two pre-mRNAs, however, MBNL and CELF proteins bind to different sites within the pre-mRNAs indicating that the antagonism is not due to a competition for a common binding site. In addition, regulation by CELF and MBNL appeared to be completely independent, as TNNT2 minigenes containing mutant CUG-BP1 binding sites still responded to MBNL1, and vice versa (Ho et al. 2004).

The patterns of misregulation for *TNNT2*, *IR*, and *CLCN-1* are consistent with increased CUG-BP1 activity and with decreased MBNL1 activity. Whether the splicing effects in DM are due primarily to loss of MBNL activity or a gain of CELF protein activity remains an open question, with evidence supporting both. Several pieces of evidence, some of which was noted above, support a role for MBNL depletion. First, MBNL proteins colocalize with CUG- and CCUG-repeat RNA foci, which is consistent with a loss of function due to sequestration (Fardaei et al. 2002; Mankodi et al. 2001; Miller et al. 2000). Second, misregulated splicing patterns and striking phenotypic similarities to DM are observed in *Mbnl* knockout mice (Kanadia et al. 2003a). Third, a recent study showed that loss of MBNL1 function was the critical event in aberrant splicing of IR in DM1 cultured cells (Dansithong et al. 2005). Fourth, of the two pre-mRNAs directly regulated by MBNL proteins (TNNT2 and IR), the splicing patterns of both alternative exons in DM tissues are consistent with a loss of MBNL activity (Ho et al. 2004).

There are also several results that support a role for increased CELF activity. First, CUG-BP1 steady state levels are increased in DM skeletal muscle and heart tissues as well as in DM cell cultures (Dansithong et al. 2005; Savkur et al. 2001; Timchenko et al. 2001a). Second, the splicing patterns of all of the three pre-mRNAs (*TNNT2*, *IR*, and *CLCN-1*) shown to be directly regulated by CUG-BP1 are consistent in DM tissues with increased CUG-BP1 activity (Charlet-B. et al. 2002b; Philips et al. 1998; Savkur et al. 2001). Third, a TNNT2 minigene expressed in DM cell cultures reproduces the splicing pattern observed for the endogenous *TNNT2* pre-mRNA in DM tissues (Philips et al. 1998). Similarly, the "DM" splicing pattern for both TNNT2 and IR minigenes can be induced in normal cells by co-expression of a plasmid containing expanded CTG repeats (Ho et al. 2004; Philips et al. 1998; Savkur et al. 2001). Importantly,

minigenes that contain mutations in the CUG-BP1 binding site are no longer responsive to CUG-BP1, to the effects in DM cell cultures, or to co-expression of CUG-repeat RNA (Philips et al. 1998; Savkur et al. 2001). These results indicate that the effects of the repeats on splicing require the CUG-BP1 binding site and suggest a direct role for CUG-BP1.

There is also evidence suggesting that sequestration of MBNL proteins is not sufficient to explain the *trans*-acting effects of CUG-repeat RNA on splicing. First, the mutated TNNT2 minigene that is not responsive to CUG-repeat RNA remains responsive to depletion of MBNL1 using siRNAs (Ho et al. 2004). The finding that a minigene that does not respond to co-expression of CUG-repeat RNA still responds to MBNL1 depletion indicates that the effects of CUG RNA on splicing involves more than MBNL depletion (Ho et al. 2004). Second, recent results indicate that MBNL colocalizes with nuclear RNA foci containing either CUG- or CAG-repeat RNA from transiently transfected plasmids. Expanded CUG-repeat RNA induces splicing changes of TNNT2 and IR minigenes when coexpressed with the minigenes but CAG repeats of equal length and expressed at comparable levels have little effect on splicing (Ho et al. 2005b). When FRAP analysis was used to determine the relative affinity of a MBNL1-GFP fusion protein for CUG- and CAG-repeat RNA foci in vivo; no differences were detected (Ho et al. 2005b). Therefore, CUG and CAG RNA appear to have similar abilities to sequester MBNL while only CUG repeats have a *trans*-dominant effect on splicing. Finally, MBNL3, also called CHCR (Cys3His CCG1-Required), was identified as an inhibitor of muscle differentiation in C2C12 cells (Squillace et al. 2002). Muscle differentiation defects observed in DM is not consistent with loss of MBNL3 function since MBNL3 sequestration in nuclear foci should favor differentiation. These results suggest that while loss of MBNL activity is likely to play a role in the splicing abnormalities observed in DM, the effects of the repeats on alternative splicing regulation appear to involve a mechanism more complex than sequestration of MBNL alone.

Accumulation of RNA foci is an important hallmark of DM (Davis et al. 1997; Jiang et al. 2004; Liquori et al. 2001; Mankodi et al. 2003; Miller et al. 2000; Taneja et al. 1995). The formation of the foci was recently shown to require MBNL as RNAi-mediated depletion of MBNL in DM1 myoblasts reduced the number of foci by 70% (Dansithong et al. 2005). It is clear that the repeat-containing RNA is pathogenic but it is not clear whether foci contain the pathogenic form of the RNA. The finding that CAG-repeat RNA forms foci, colocalizes with MBNL but does not alter splicing strongly suggest that foci formation and the potential to sequester MBNL alone is not sufficient for misregulated alternative splicing. Additional support for the inconsistency between toxicity and foci formation comes from a recent report showing that expression of 162 CTG repeats in the 3′ UTR of a reporter gene formed foci in *Drosophila* tissues without inducing pathology, suggesting that foci formation was not toxic to *Drosophila*

(Houseley et al. 2005). Similarly, foci formation by RNAs containing only CUG repeats is not sufficient to induce muscle-differentiation defects in the C2C12 cell line (Amack and Mahadevan 2001). In summary, these results strongly suggest that foci formation alone is not pathogenic.

3.4.3 Sequestration of Other RNA Binding Proteins

In addition to MBNL proteins, the splicing regulators hnRNP H and F colocalize with CUG foci in neurons of DM1 patient brain samples (Jiang et al. 2004). Neuron-specific c-*src N1* exon is regulated by hnRNP F (Min et al. 1995) and hnRNP H regulates *NF-1* exon 3, thyroid stimulating hormone beta subunit (*TSH beta*) genes (Buratti et al. 2004), HIV-1 tev-specific exon 6D (Caputi and Zahler 2002) and beta tropomyosin (Chen et al. 1999). The relevance of hnRNP H and hnRNP F colocalization with RNA foci is not clear since splicing of *c-src* is not disrupted in neurons (Jiang et al. 2004).

Double-stranded-RNA-dependent protein kinase R (PKR), is activated by double-stranded RNA as a response to viral infections (Williams 2001). Activation of PKR inhibits translation by phosphorylation of translation initiation factor eIF2 alpha (Clemens 2001). PKR was identified as one of the RNA-binding proteins that bind to double-stranded CUG repeats, and PKR is activated by CUG-repeat expression in vitro (Tian et al. 2000). Further studies using mouse models; however, indicated that PKR is not crucial to disease pathogenesis. Neither myotonia nor histological changes were altered in HSA_{250} mice on a PKR–/– or PKR–/+ background, suggesting that PKR is unlikely to be relevant to DM pathogenesis (Mankodi et al. 2003).

3.4.4 Transcriptional Interference

The toxicity of expanded CUG repeats is proposed to result from sequestering transcription factors similar to a mechanism of pathogenesis for polyglutamine expansions (Ebralidze et al. 2004). The transcription factors Sp1 and retinoic acid receptor gamma were found to be recruited to the expanded CUG repeats and depleted from the active chromatin correlating with reduced expression of several genes including *CLCN-1* (encodes for ClC-1 protein) detected by real time RT-PCR analysis (Ebralidze et al. 2004). In addition to alternative splicing misregulation and likely downregulation by NMD, reduced transcription of *CLCN-1* mRNA might also contribute to loss of ClC-1 protein and myotonia. In contrast to the expectation that transcription factors are sequestered with CUG-repeat RNA, however, Jiang and colleagues could not detect Sp1 or retinoic acid receptor gamma associated with RNA foci in brain tissues of DM1 patient cells by immunofluorescence (Jiang et al. 2004).

3.4.5 Muscle Differentiation Defects and Altered Translation Regulation

Muscle weakness and wasting are the major causes of mortality in individuals with DM1 (Harper 2001). Delays or defects in muscle differentiation have been proposed as the major factors that lead to muscle weakness and wasting. In culture, normal muscle cells proliferate in growth medium; upon removal of growth factors, the cell cycle is inhibited and the cells enter the differentiation pathway. Differentiating cells fuse into multi-nucleated myotubes and express muscle-specific genes (Olson 1992). A defect in muscle differentiation has been observed both in individuals with congenital and adult onset DM1 (Furling et al. 2001; Timchenko et al. 2001b). Morphological and histochemical studies revealed developmental defects in satellite cells from individuals with CDM expressing 2300 CTG repeats. Myoblast fusion was less complete in cells with nuclear RNA foci suggesting a defect in myogenic differentiation associated with CUG-repeat RNA. In addition to poor muscle differentiation, satellite cells had a reduced life span and proliferation capacity in culture (Furling et al. 2001).

Myoblasts from individuals with DM1 were unable to withdraw from the cell cycle when stimulated to differentiate (Timchenko et al. 2001b). In C2C12 cells, four- to ten-fold constitutive overexpression of the human *DMPK* 3′UTR inhibited muscle differentiation. The inhibitory activity was mapped to a 239-nucleotide region located upstream of the CTG repeats (Sabourin et al. 1997). Recently, the toxic effects of *DMPK* 3′ UTR was reproduced in transgenic mice overexpressing *DMPK* 3′-UTR with wild type (11) or expanded (91) CTG repeats (Storbeck et al. 2004). Both expanded and wild-type CTG-repeat-expressing mice displayed muscle atrophy supporting the previous findings that mainly *DMPK* 3′ UTR is responsible for muscle differentiation defects (Storbeck et al. 2004). Myoblast cultures from these animals showed reduced fusion, but disruption of muscle differentiation was worse in the presence of expanded CTG repeats. These results suggest that the *DMPK* 3′UTR was sufficient for defects in muscle differentiation. On the other hand, in C2C12 cell lines stably expressing the normal *DMPK* 3′UTR, muscle differentiation was not disrupted. Only *DMPK* 3′UTR expressing 200 CTG repeats inhibited C2C12 myoblast differentiation (Amack et al. 1999). These results suggest that repeats are necessary for inhibition of muscle differentiation. MyoD was identified as a target for the inhibitory effects of *DMPK* 3′UTR with expanded CTG repeats during C2C12 differentiation as well as during DM1 myoblast differentiation (Amack et al. 2002; Timchenko et al. 2001b). Specifically, expression of the *DMPK* 3′UTR with 200 CTG repeats severely reduced MyoD levels (Amack et al. 2002) and reduced levels of MyoD were detected in myoblasts from individuals with DM1 (Timchenko et al. 2001b).

The p21 protein, which is an important regulator of cell cycle progression and muscle differentiation, was identified as a downstream target for the muscle differentiation defects induced by expanded CTG repeats (Timchenko et al. 2001b). CUG-BP1 was shown to enhance p21 translation by binding to GCN repeats in the 5′ UTR of p21 mRNA (Timchenko et al. 2001b). Accumulation of CUG-BP1 in the nuclei of DM cells resulted in reduced levels of p21 translation leading to reduced differentiation of these cells. These results suggest that reduced MyoD and p21 are directly involved in muscle development abnormalities seen in CDM or defects in muscle regeneration seen in DM1.

The molecular mechanisms for muscle weakness and wasting in DM2 patients are not clear since there is no involvement of *DMPK* 3′UTR in DM2. To date, none of the transgenic or knockout mouse models have reproduced a clear progressive muscular dystrophy phenotype as observed in individuals with DM1 (Berul et al. 2000; Kanadia et al. 2003a; Klesert et al. 2000; Mankodi et al. 2000; Mounsey et al. 2000; Reddy et al. 1996; Sarkar et al. 2000; Seznec et al. 2001; Timchenko et al. 2004). There are several possibilities for the lack of this phenotype in mouse models. First, mice might not show the same phenotype as humans due to physiological differences. Second, there is no mouse model that expresses more than 300 CTG repeats. Longer repeats might have more severe effects in muscle development. Alternatively, higher levels of expression of shorter repeats could also show a more severe phenotype. Finally, the mouse life span might not be long enough for the disease to worsen. In the future, it will be important to have animal models that could represent dystrophy seen in DM to better understand the molecular mechanisms involved and find better ways to treat patients.

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