

# Developmental Insights Into the Pathology of and Therapeutic Strategies for DM1: Back to the Basics

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Myotonic Dystrophy type 1 (DM1), the most prevalent adult onset muscular dystrophy, is a trinucleotide repeat expansion disease caused by CTG expansion in the 3'-UTR of DMPK gene. This expansion results in the expression of toxic gain-of-function RNA that forms ribonuclear foci and disrupts normal activities of RNA-binding proteins belonging to the MBNL and CELF families. Changes in alternative splicing, translation, localization, and mRNA stability due to sequestration of MBNL proteins and up-regulation of CELF1 are key to DM1 pathology. However, recent discoveries indicate that pathogenic mechanisms of DM1 involves many other factors as well, including repeat associated translation, activation of PKC-dependent signaling pathway, aberrant polyadenylation, and microRNA deregulation. Expression of the toxic repeat RNA culminates in the developmental remodeling of the transcriptome, which produces fetal isoforms of proteins that are unable to fulfill the physiological requirements of adult tissues. This review will describe advances in the understanding of DM1 pathogenesis as well as current therapeutic developments for DM1. *Developmental Dynamics* 244:377–390, 2015. © 2014 Wiley Periodicals, Inc.

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## Introduction

The discovery of the genetic mutation responsible for fragile X syndrome and spinal and bulbar muscular atrophy more than 20 years ago revealed the nature of repeat expansion diseases, and changed the way genetically inherited diseases were understood. Repeat expansion diseases include trinucleotide, tetranucleotide, pentanucleotide, and hexanucleotide repeat expansions. Phenotype severity and age of onset vary depending on the expansion size. Although the genetic basis of these diseases is similar, the pathological mechanisms can differ significantly. Repeat expansion in the 5'-UTR (such as in fragile X syndrome) can lead to inhibition of gene expression, while repeat expansion in the 3'-UTR can result in expression of toxic gain-of-function RNAs (such as in myotonic dystrophy type 1). Repeat expansion in the coding region of genes, such as that in Huntington's disease, can result in the expression of toxic protein that interferes with cellular functions. In this review, we focus on pathogenesis and therapeutic development for myotonic dystrophy type 1.

Myotonic Dystrophy type 1 (DM1) is the second most common cause of muscular dystrophy after Duchenne muscular dystrophy and the most common cause of adult onset muscular dystrophy with a worldwide incidence of 1 in 8,500 (Day and Ranum, 2005). It is autosomal dominantly inherited and affects multiple tissues with symptoms including muscle hyperexcitability (myotonia), progressive muscle wasting, cardiac arrhythmias, insulin resistance, gastrointestinal dysfunctions, posterior iridescent cataracts, and neuropsychiatric disturbances (Harper, 2001). Similar to other trinucleotide expansion diseases, disease severity and age of onset in DM1 approximately correlates with the size of the expansion. Pathology of DM1 stems from an expanded CUG repeat (CUG<sup>exp</sup>) found at the 3'-UTR of the dystrophin myotonia protein kinase (*DMPK*) gene (Brook et al., 1992; Caskey et al., 1992), which forms stable hairpin loops (Napierala and Krzyzosiak, 1997) and causes *trans* dominant effects (Fig. 1). Normal individuals have from 5 to 34 repeats. Symptoms have been identified in individuals with 50 repeats, whereas large repeat sizes (>1,000) typically associate with the worse form of disease (Brook et al., 1992; Day and Ranum, 2005; Cho and Tapscott, 2007). The congenital form is much more severe and is characterized by general muscle hypotonia and respiratory distress at birth, as well as delayed motor and cognitive development.

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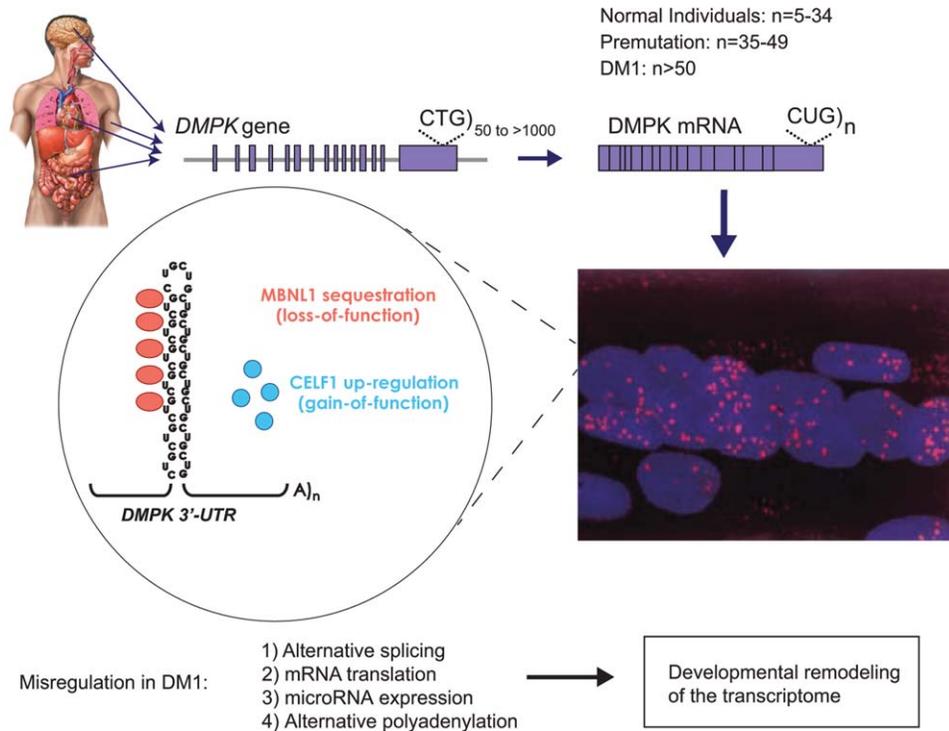
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**Fig. 1.** Schematic model of the genetic mutation responsible for DM1 pathogenesis. DM1 is a multi-systemic disease that affects the heart, skeletal muscle, gastro-intestinal and central nervous systems. CUG repeat expansion at the 3'-UTR of the *DMPK* gene causes formation of ribonuclear foci that sequester MBNL1 and up-regulates CELF1 through microRNA misregulation and PKC activation. Misregulation of these RNA-binding proteins affects a wide range of post-transcriptional and translational events, ultimately resulting in developmental remodeling of the transcriptome. Human Diagram used here was obtained from [http://www.nofas-uk.org/OnlineCourse/IMAGES/organ\\_LGE.jpg](http://www.nofas-uk.org/OnlineCourse/IMAGES/organ_LGE.jpg). Ribonuclear foci image is reproduced from Davis et al. (1997) with permission of the publisher.

With the discovery of the repeats, several hypotheses initially emerged regarding the possible mechanisms responsible for the observed DM1 pathologies. One such hypothesis was the *DMPK* haploinsufficiency model, where DM1 pathology was thought to be a result of CUG-repeat induced inhibition of *DMPK* protein expression. However, *Dmpk* knockout mice could not reproduce the major DM1 symptoms, such as myotonia, muscle wasting, and splicing misregulation (Jansen et al., 1996; Berul et al., 1999). Another hypothesis proposed that the presence of CUG repeats could affect expression of genes adjacent to *DMPK* gene. Although the adjacent gene *SIX5* does have reduced expression in DM1 patients (Thornton et al., 1997), *Six5* knockout mice do not exhibit any muscle pathology (Klesert et al., 2000). Furthermore, the CUG<sup>exp</sup> RNA was discovered to form stable hairpin structures that aggregate into ribonuclear foci (Davis et al., 1997; Napierala and Krzyzosiak, 1997). Transgenic overexpression of untranslated CUG repeats in the mouse skeletal muscle provided the essential evidence for the toxicity of expanded RNA (Mankodi et al., 2000). Remarkably, forced expression of 250 CUG repeats under human skeletal actin promoter (HSA<sup>LR</sup> mice) reproduces myotonia, myopathy, ribonuclear foci accumulation, MBNL1 sequestration, as well as splicing misregulation found in DM1 patients. Since then multiple labs have generated tissue-specific constitutive and inducible mouse models expressing different lengths and contexts of CUG<sup>exp</sup> RNA, which have aided in discovery of much of the currently known DM1 pathology mechanisms (Gourdon et al., 1997; Seznec et al., 2000; Mahadevan et al., 2006; Wang et al., 2007; Orengo et al., 2008). The advantages and disadvantages of each of these models in recapitulating

DM1 pathology are reviewed elsewhere (Gomes-Pereira et al., 2011).

The discovery provided the foundation for the paradigm based on "RNA gain of toxic function." According to this paradigm, the primary pathology of DM1 can be traced to the misregulation of two RNA-binding protein families: muscleblind-like (MBNL) and CUGBP and Elav-like family members (CELF). The molecular mechanisms by which CUG<sup>exp</sup> RNA results in the disease include: (1) the accumulated CUG<sup>exp</sup> RNA folds into a hairpin-like structure (Moers et al., 2005) and binds to MBNL proteins with high affinity, causing their depletion and loss-of-function in the nucleoplasm (Kanadia et al., 2003a); (2) CUG<sup>exp</sup> RNA activates the protein kinase C pathway and suppresses expression of specific microRNAs culminating in up-regulation of CELF1, resulting in its gain-of-function (Kuyumcu-Martinez et al., 2007; Wang et al., 2007; Kalsotra et al., 2014). Both MBNL1 and CELF1 are required for normal splicing regulation during development (Lin and Ying, 2006; Kalsotra et al., 2008) (Fig. 1). Disruption of their functions by CUG<sup>exp</sup> RNA leads to missplicing of many genes some of which (*CLCN1*, *BIN1*, *IR*, *PKM*, and *TNNT2*) explain the prominent features of the disease. However, it is becoming increasingly clear that expanded CUG repeats cause widespread signaling, transcriptional and post-transcriptional perturbations that are not wholly driven by MBNL1 and/or CELF1.

### Loss of Function of MBNL Proteins

MBNL proteins were first characterized as factors involved in the pathogenesis of DM1 but were subsequently shown to be direct

regulators of alternative splicing (Kanadia et al., 2003a; Ho et al., 2004). There are three MBNL paralogs in mammals. MBNL1 and MBNL2 are expressed across many tissues, including brain, heart, muscle, and liver, whereas MBNL3 is expressed primarily in placenta (Kanadia et al., 2003b). The observation that MBNL1 was most highly expressed in heart and skeletal muscle corroborated the fact that the most severe DM1 phenotypes are manifested in these tissues. Further experimentation in DM1 myoblasts showed that MBNL1 is depleted from the nucleoplasm and is extensively sequestered on to the *DMPK* ribonuclear inclusions (Miller et al., 2000). Moreover, independent of the repeat expansion, *Mbnl1*<sup>Δ3/Δ3</sup> mice (*Mbnl1* knockouts) reproduce many of the key features of DM1 including myotonia, myopathy, cataracts, and misregulation of alternative splicing (Kanadia et al., 2003a; Du et al., 2010). MBNL2 and MBNL3 also co-localize with the CUG<sup>exp</sup> RNA foci resulting in loss-of function of all three paralogs (Fardaei et al., 2002). Muscleblind is also sequestered in a fly model of DM1 and the muscle and eye phenotype in these flies can be rescued by MBNL1 over-expression (de Haro et al., 2006). Recently, MBNL proteins were shown to play necessary roles in normal erythroid differentiation and proliferation (Cheng et al., 2014) as well as in suppression of embryonic stem cell pluripotency (Han et al., 2013).

Mammalian MBNL proteins contain four-tandem zinc finger domains each composed of three cysteine and one histidine residue that coordinate zinc atoms to bind single-stranded RNA in a sequence-specific manner (Pascual et al., 2006). An initial study showed that MBNL1 strongly binds to CUG, CCUG, and CAG repeats (Kino et al., 2004). Genome-wide analyses, together with crystal structure and biochemical studies revealed that MBNL1 preferentially recognizes a pyrimidine-rich YGCY motif (Y stands for C or U) within pre-mRNA (Castle et al., 2008; Teplova and Patel, 2008; Du et al., 2010; Goers et al., 2010; Wang et al., 2012). The zinc finger motifs lie in between the complementary base pairs of the RNA hairpin such that MBNL1 interactions with the RNA are stabilized through stacking of aromatic and arginine residues with guanine and cytosine bases and a network of hydrogen bonds (Teplova and Patel, 2008; Konieczny et al., 2014).

Human *MBNL1* consists of twelve exons, six of which are alternatively spliced, resulting in the expression of seven known *MBNL1* variants (Fardaei et al., 2002). It has been shown that alternative splicing of *MBNL1* can modulate its affinity towards CUG<sup>exp</sup> RNA (Kanadia et al., 2003b). Inclusion of exon 3 increases MBNL1's affinity for CUG repeats, whereas inclusion of exon 7 enhances both MBNL1 self-dimerization and its binding to the repeat RNA. Furthermore, exon 7 is required for MBNL1 nuclear localization, as it is part of a biNLS and conNLS (Kino et al., 2014). Intriguingly, MBNL1 represses its own exon 7 splicing in a dose-dependent manner such that loss of MBNL1 activity results in increased exon 7 inclusion and thereby MBNL1 nuclear retention. In addition to their nuclear role in splicing regulation, MBNL proteins also have significant cytoplasmic expression and can regulate mRNA stability (Osborne et al., 2009; Du et al., 2010; Masuda et al., 2012) and localization (Adereth et al., 2005; Wang et al., 2012). Furthermore, MBNL1 can regulate the circularization of its own transcript (Ashwal-Fluss et al., 2014). The flanking introns of exon 2 in drosophila muscleblind (MBL) transcripts contain numerous MBL-binding sites such that overexpression of MBL isoform A and C increases biogenesis of circMbl expression by 13-fold, whereas knockdown of MBL result in a 2-fold decrease in circMbl. This phenomenon is conserved in

humans suggesting MBNL1 is capable of regulating its own mRNA expression via circularization (Ashwal-Fluss et al., 2014).

Direct comparison of transcriptome changes between HSA<sup>LR</sup> and *Mbnl1*<sup>Δ3/Δ3</sup> mouse models showed that loss of MBNL1 accounts for greater than 80% of the splicing pathology due to CUG<sup>exp</sup> RNA (Du et al., 2010). In a combined RNA-seq and CLIP-seq study of MBNL targets in muscle, myoblasts, brain, and heart, MBNL1 and MBNL2 were found to co-regulate certain targets suggesting their splicing functions may be interchangeable (Wang et al., 2012). The extent of DM1 symptoms, however, may vary depending on tissue context and the degree to which MBNL1 and MBNL2 levels are altered as well (Lee et al., 2013). For instance, only a few splicing perturbations were found in the brain of *Mbnl1*<sup>Δ3/Δ3</sup> mice compared to hundreds found in skeletal muscle (Du et al., 2010; Suenaga et al., 2012). Conversely, *Mbnl2*<sup>Δ2/Δ2</sup> knockout mice manifest a number of DM1-related central nervous system abnormalities including irregular REM sleep propensity and deficits in spatial memory (Charizanis et al., 2012). Moreover, loss of MBNL2 in the brain triggered misregulation of hundreds of DM1-associated splicing events highlighting tissue-specific requirement of individual MBNL paralogs and their effects in DM1 pathology. This is consistent with the observation that MBNL2 has higher expression in the brain than MBNL1 (Kanadia et al., 2003b).

## Gain of CELF1 Activity

CELF1, also called CUG binding protein 1 (CUGBP1), was initially discovered in a screen from HeLa cell proteins that bound the CUG repeat motif (Timchenko et al., 1996). However, it was later found that CELF1 prefers to bind to the base of the CUG hairpin structure and that its affinity for the *DMPK* transcript is not proportional to the CUG repeat size (Michalowski et al., 1999). Importantly, the localization pattern of CELF1 in DM1 and normal cells is identical, indicating that unlike MBNL proteins CUG<sup>exp</sup> RNA does not sequester CELF proteins. On the contrary, several studies demonstrated that CELF1 protein but not mRNA steady state levels are increased 2- to 4-fold in DM1 cells and tissues (Timchenko et al., 1996; Philips et al., 1998; Savkur et al., 2001). CELF1 upregulation in DM1 occurs via two independent mechanisms: CELF1 protein stabilization mediated by its hyperphosphorylation by PKC (Kuyumcu-Martinez et al., 2007); and through reduced levels of miR-23a/b (Kalsotra et al., 2014), which normally suppress CELF1 protein translation in heart tissue (Kalsotra et al., 2010). The signaling pathway that activates PKC-mediated hyperphosphorylation of CELF1 in response to CUG<sup>exp</sup> RNA remains to be determined.

Increased CELF1 proteins levels are thought to significantly contribute to the overall DM1 pathogenesis. For instance, transgenic overexpression of CELF1 in striated muscle tissues results in defects in muscle histology and physiology that recapitulate DM1 phenotypes. Skeletal muscle- and heart-specific overexpression of CELF1 in mice reproduce splicing misregulation as is seen in DM1 patients (Ho et al., 2005). Heart-specific CELF1 overexpression results in dilated cardiomyopathy and conduction abnormalities similar to DM1 heart phenotype (Koshelev et al., 2010). Furthermore, skeletal muscle-specific CELF1 overexpression results in centrally nucleated myofibers, myofiber atrophy, and impaired muscle performance (Ward et al., 2010). Since muscle wasting is not seen in *Mbnl1*<sup>Δ3/Δ3</sup> mice, CELF1 upregulation is thought to promote the muscle wasting phenotype observed in DM1.

CELF proteins share a common domain structure containing three RNA recognition motifs (RRMs) and a unique linker region called the “divergent domain,” which separates RRM2 and RRM3 (Ladd et al., 2001). There are six CELF paralogues in mammals and most are widely expressed with the highest expression in striated muscle and brain (Good et al., 2000; Ladd et al., 2001; Ladd and Cooper, 2004). CELF1 participates in multiple RNA-processing events in the nucleus and cytoplasm, including alternative pre-mRNA splicing, mRNA editing, decay, deadenylation, and translation (Dasgupta and Ladd, 2012). Muscle cells from DM1 patients can exhibit delayed myogenic differentiation due to their reduced ability to withdraw from cell cycle, which may be due to altered translation of the cell cycle inhibitor *P21* or the myogenic transcription factor *MEF2A* by CELF1 (Timchenko et al., 2001; Timchenko et al., 2004). However, a recent study failed to replicate the result that DM1 patient cell lines are impaired to undergo *in vitro* myogenesis (Loro et al., 2010).

CELF1 affinity for select mRNAs can be modulated by phosphorylation via AKT or cyclinD3-CDK4/6 (Salisbury et al., 2008). AKT phosphorylates CELF1 in proliferating myoblasts, which increases CELF1’s affinity for cyclinD1 mRNA. During normal myoblast differentiation, cyclinD3-cdk4/6 phosphorylates CELF1, which leads to increased CELF1 interaction with *p21* and *C/EBP $\beta$*  mRNAs. Myoblasts from DM1 patients have increased interaction between CELF1 and AKT, as well as reduced cyclinD3-CDK4/6 levels during differentiation. CELF1 also destabilizes mRNAs through binding to the GU-rich elements in their 3’-UTRs (Vlasova et al., 2008; Lee et al., 2010). This is likely due to its recruitment of poly(A) specific ribonuclease, which promotes deadenylation of target transcripts (Moraes et al., 2006). Additionally, CELF1 knockdown stabilizes many of its directly bound target transcripts, which are particularly enriched for functions related to muscle differentiation, cell cycle, apoptosis, and RNA processing (Zhang et al., 2008; Rattenbacher et al., 2010).

## Developmental Reprogramming of the Transcriptome

Alternative pre-mRNA splicing is a primary source of transcriptome complexity in humans as it allows multiple transcripts with potentially different functions to be produced from a single gene (Nilsen and Graveley, 2010). More than 90% of human genes are alternatively spliced and most are dynamically regulated in a cell-type or developmental stage-specific manner (Castle et al., 2008; Pan et al., 2008; Wang et al., 2008). These different isoforms can considerably impact cellular functions such that regulation of alternative splicing determines specific physiological outcomes (Kalsotra and Cooper, 2011). Splicing is controlled by RNA-binding proteins (RBPs), which bind to the “core” and “auxiliary” elements present on pre-mRNAs and thus influence the spliceosome assembly near the 5’ and 3’ splice sites (Braunschweig et al., 2013). Approximately 50 mammalian RBPs have been characterized and shown to directly regulate splicing by binding to pre-mRNAs (Gabut et al., 2008; Nilsen and Graveley, 2010). Many of these act as tissue- and cell type-specific splicing factors. Cooperative or opposing effects of these factors often control individual splicing events. At the same time, hundreds of splicing events can be sensitive to changes in the level of a single splicing factor (Kalsotra and Cooper, 2011).

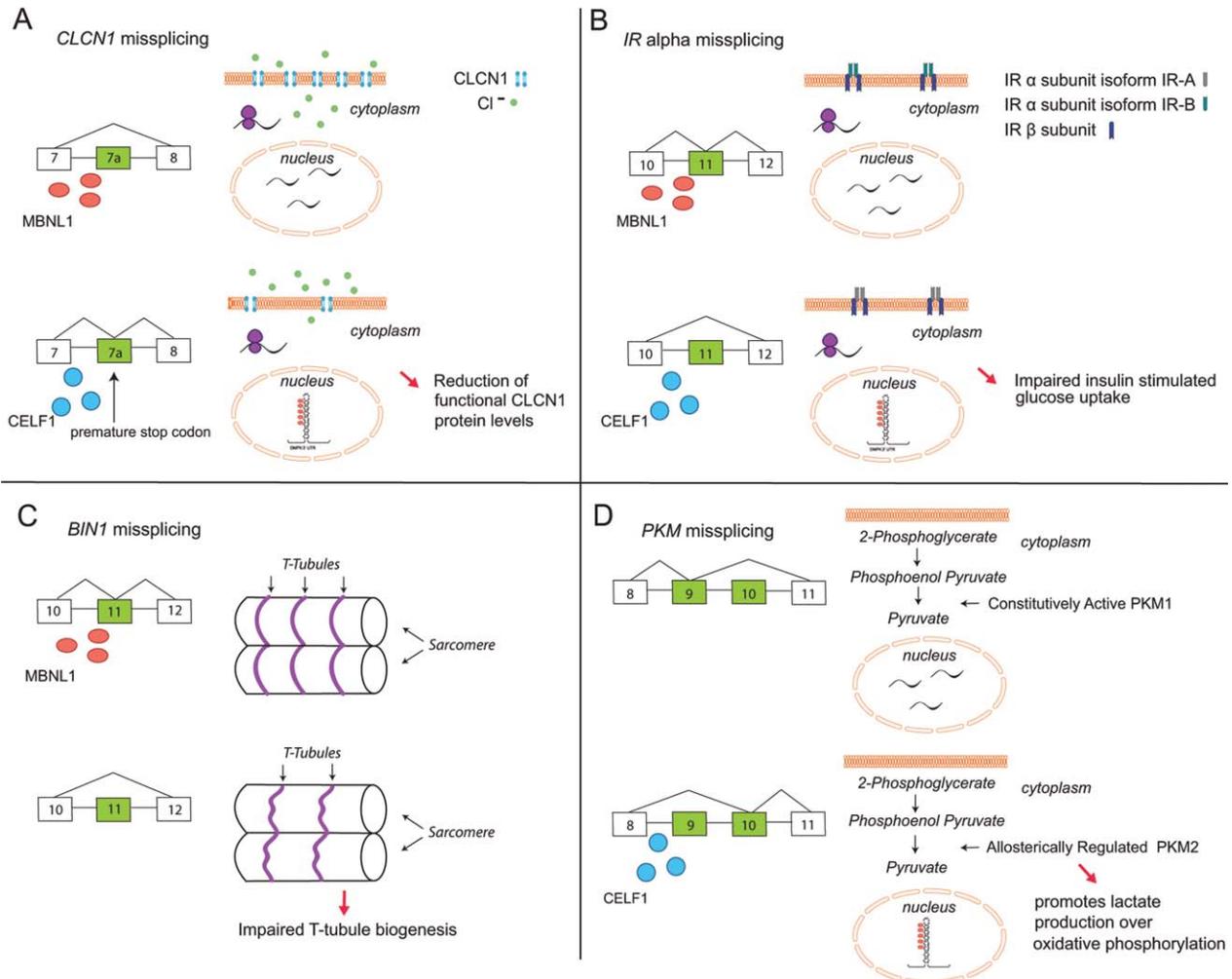
Splicing misregulation is a cardinal feature of DM1. The misregulated events are normally developmentally regulated, and in

DM1, undergo an adult-to-embryonic switch in splicing patterns. Many of these embryonic isoforms are unable to fulfill the proper adult tissue requirements resulting in specific defects that contribute to the overall disease pathogenesis (discussed in the next section). It is critical to note that the splicing defects in DM1 do not arise due to *cis* mutations within exons or splice sites at the intron-exon boundaries. Instead, they are a consequence of altered activities of the *trans* factors, MBNL and CELF proteins, which regulate alternative splicing transitions during normal striated muscle development. For some pre-mRNAs whose splicing is disrupted in DM1, CELF1 and MBNL1 regulate them antagonistically (Savkur et al., 2001; Charlet et al., 2002; Ho et al., 2004). Interestingly, antagonism is not via competition for the same binding site as CELF1 and MBNL1 both bind and regulate splicing independently through distinct *cis* regulatory sequences.

Postnatal loss of CELF and gain in MBNL activity is part of the normal developmental program that facilitates embryonic-to-adult switch in splicing patterns of a large number of genes. MBNL1 activity in striated muscle increases postnatally via at least two different mechanisms. In skeletal muscle, MBNL1 becomes more nuclear through a change in its localization (Lin et al., 2006), whereas in cardiac muscle, the protein levels increase by 4-fold during postnatal development (Kalsotra et al., 2008). MBNL proteins bind core YGCY intronic motifs surrounding many developmentally regulated alternative exons (Goers et al., 2010; Grammatikakis et al., 2011). These intronic motifs fold into hairpins, which resemble the GC-rich secondary structure of CUG<sup>exp</sup> RNA (Warf and Berglund, 2007; Yuan et al., 2007). In contrast to MBNL1, CELF proteins are post-transcriptionally downregulated during postnatal development (Ladd et al., 2001; Kalsotra et al., 2008). The downregulation is mediated by microRNA-mediated translation repression and protein destabilization by dephosphorylation (Kuyumcu-Martinez et al., 2007; Kalsotra et al., 2010). Normally CELF1 is hyperphosphorylated by PKC $\alpha$ /  $\beta$ II during embryonic stage, but a developmentally regulated reduction in PKC $\alpha$ /  $\beta$ II during heart development mediates a decrease in steady state levels of CELF1.

CELF and MBNL proteins regulate the developmental splicing transitions independently, synergistically, as well as antagonistically (Kalsotra et al., 2008; Giudice et al., 2014). This shift occurs as part of a normal transition of cells adapting to the needs of the maturing organ. Interestingly, CELF and MBNL binding motifs have a positional effect on splicing such that binding in the upstream intron correlates with exon skipping while binding in the downstream intron correlates with increased exon inclusion (Kalsotra et al., 2008; Du et al., 2010). In DM1, the postnatal regulation of MBNL1 and CELF1 is reversed, which reprograms the developmentally regulated splicing network towards the embryonic state. Such transcriptome changes restructure the adult tissue function mimicking earlier developmental stages. For example, T-tubule organization in adult cardiomyocytes with elevated CELF1 levels resembles immature early postnatal structures (Giudice et al., 2014).

In addition to alternative splicing, alternative polyadenylation (APA) also contributes in expanding the RNA transcript diversity required for mammalian tissue development (Di Giammartino et al., 2011). Alternative 3’UTR’s formed through APA alter interactions of transcripts with *trans*-acting factors to modulate their translation, localization, and turnover (Tian and Manley, 2013). Misregulation of developmentally regulated APA due to MBNL1 sequestration in DM1 tissues was recently discovered (Batra et al.,



**Fig. 2.** Examples of missplicing in DM1 with functional consequences. **A:** Missplicing of *CLCN1* results in the NMD-mediated degradation of the transcript due to the presence of a premature stop codon in retained intron 2 (not shown) and/or exon 7a. This causes a decrease in functional Clcn1 protein, which manifests as myotonia in DM1 patients. **B:** Missplicing of *IR* results in expression of the IR-A isoform that has lower signaling capability and lower tyrosine kinase activity. This causes impairment of insulin-stimulated glucose uptake in DM1 patients. **C:** Missplicing of *BIN1* results in an isoform that cannot bind phosphoinositides and tubulate membranes, which disrupts T-tubule biogenesis and causes muscle weakness. **D:** Missplicing of *PKM* results in an allosterically regulated isoform that promotes lactate production over oxidative phosphorylation. Type 1 myofiber specific PKM2 upregulation disrupts energy metabolism, and may be involved in the muscle-wasting phenotype in DM1 patients.

2014). Mbnl1/Mbnl2 double knockout (DKO) and DKO/Mbnl3 knockdown (DKO/3KD) mouse embryonic fibroblasts showed a large-scale and predominant shift towards the proximal polyadenylation sites. A large proportion of these events was developmentally regulated (Batra et al., 2014). Importantly, 66% of genes with altered polyadenylation in HSA<sup>LR</sup> mice reverted back towards the embryonic pattern such that the distal to proximal ratio of APA site selection in adult HSA<sup>LR</sup> muscle was similar to that of wild-type neonatal muscles. Similar misregulation of APA was observed in human DM1 skeletal muscle (Batra et al., 2014).

Developmental reprogramming of the transcriptome extends to miRNA expression as well. Of the 54 miRNA that are differentially expressed in the heart between DM1 and control mice, 45 are developmentally regulated (Kalsotra et al., 2014). Forty-two of these miRNAs are normally upregulated during heart development but are strongly reduced upon CUG<sup>exp</sup> expression in adult DM1 mice. This adult to embryonic shift in miRNA expression pattern contributes to the remodeling of the transcriptome

towards a more embryonic-like pattern. One such consequence is the reduced expression of miR-23a/b that results in an increase in expression of CELF1 (Kalsotra et al., 2010).

## Pathogenic Consequences of Splicing Defects

Some of the phenotypes in DM1 can be attributed to misregulation of alternative splicing. Here we describe key examples that have direct impact on the disease symptoms (Fig. 2).

### Missplicing of *CLCN1*

Myotonia is defined as excessive muscle excitability upon voluntary activation followed by repetitive action potentials, stiffness, and delayed relaxation. Myotonia in DM1 occurs due to abnormal splicing of the muscle-specific chloride channel (*CLCN1*) and

reduced conductance of chloride ions in the sarcolemma (Charlet et al., 2002; Mankodi et al., 2002). In DM1 individuals, *CLCN1* transcripts are aberrantly spliced to include intron 2 or exon 7a both of which contain premature stop codons (Lueck et al., 2007a). These splice isoforms undergo nonsense-mediated decay resulting in severe reduction in the functional CLCN1 protein (Charlet et al., 2002; Mankodi et al., 2002) (Fig. 2A). Splicing of *CLCN1* is regulated by both MBNL1 and CELF1. For instance, overexpression of CELF1 in cell culture is sufficient to increase intron 2 inclusion (Lueck et al., 2007a), whereas *Mbnl1*<sup>Δ3/Δ3</sup> mice exhibit myotonia and aberrant *Clcn1* splicing due to increased inclusion of exon 7a (Kanadia et al., 2003a; Lueck et al., 2007b). Moreover, MBNL1 overexpression in a DM1 mouse model has been shown to correct *Clcn1* splicing to the adult pattern and rescue myotonia (Kanadia et al., 2006). Similarly, targeting the 3' splice site of *Clcn1* to inhibit exon 7a inclusion restored the full-length reading frame, increased the expression of CLCN1 protein on the surface membrane, eliminated chloride channelopathy, and suppressed myotonic discharges in two different mouse models of DM1 (Wheeler et al., 2007).

### Missplicing of IR

The insulin resistance phenotype in DM1 (Moxley et al., 1978) is attributed to the aberrant splicing of Insulin Receptor (*IR*). DM1 patients express the exon 11 skipped isoform of *IR* $\alpha$  subunit in skeletal muscle (Savkur et al., 2001), which has a lower signaling capability and a lower tyrosine kinase activity compared to the normal full-length isoform (Kellerer et al., 1992) (Fig. 2B). Expression of CUG<sup>exp</sup> RNA in cell culture is sufficient to induce this splicing switch resulting in impairment of insulin-stimulated glucose uptake and incorporation into glycogen (Savkur et al., 2001). During normal development, exon 11 splicing of *IR* is antagonistically regulated by CELF1 and MBNL1. CELF1 represses and MBNL1 enhances the inclusion of exon 11 via direct binding to the respective intronic silencer and enhancer sequences in *IR* pre-mRNA (Ho et al., 2004; Sen et al., 2010; Grammatikakis et al., 2011).

### Missplicing of BIN1

Bridging integrator 1 (*BIN1*), also known as amphiphysin 2, is a nucleocytoplasmic adapter protein involved in the organization of the T-tubule network, a specialized membrane structure that supports excitation-contraction coupling in the skeletal muscle (Razzaq et al., 2001). Exon 11 skipping of *BIN1* in DM1 skeletal muscle results in an isoform that cannot bind phosphoinositides and tubulate membranes (Lee et al., 2002; Fugier et al., 2011) (Fig. 2C). Expression of CUG repeats or MBNL1 depletion represses inclusion of exon 11 of *BIN1* minigene whereas forced skipping of this exon in wildtype mice disrupts T-tubule biogenesis and produces significant reduction in muscle strength. Remarkably, the tubular membrane defects in DM1 muscle cells can be restored upon expression of the normal isoform of *Bin1* indicating missplicing contributes to the muscle weakness phenotype in DM1 (Fugier et al., 2011).

### Missplicing of PKM

Pyruvate kinase M (*PKM*) gene encodes a key metabolic enzyme that produces pyruvate during glycolysis. Alternative splicing of

mutually exclusive exons 9 and 10 in *PKM* transcript determines the fate of glucose metabolism in proliferating versus differentiated cells. Exon 9 inclusion gives rise to the constitutively active PKM1 isoform and exon 10 inclusion produces the allosterically regulated PKM2 isoform (Noguchi et al., 1986; Christofk et al., 2008a; Luo and Semenza, 2012). Proliferating cells express PKM2 isoform, which favors lactate production over oxidative phosphorylation whereas differentiated cells express PKM1 isoform that promotes oxidative phosphorylation. PKM2 can additionally bind to the phosphotyrosine residues, which release its allosteric activator, fructose 1–6 bisphosphate (Christofk et al., 2008b). This causes a transient inhibition of PKM activity in dividing cells resulting in accumulation of glycolytic intermediates that may be used for other biosynthetic processes (Fig. 2D).

Normally, in skeletal muscle, *PKM2* is expressed at the initial embryonic stages, but is replaced by *PKM1* towards the late embryonic and early postnatal stages (Noguchi et al., 1986). In DM1 skeletal muscle, *PKM2* mRNA increases to 30% of total *PKM* transcripts compared to 7% in normal tissue (Gao and Cooper, 2013). *PKM2* is specifically upregulated in type 1 myofibers, which is the fiber type that is susceptible to wasting in DM1 (Fig. 2D). Notably, MBNL depletion has minimal effect on *PKM* splicing in cultured muscle cells. However, CELF1 overexpression in skeletal muscle upregulates *PKM2* suggesting *PKM* splicing switch in DM1 is likely due to increased CELF1 levels (Gao and Cooper, 2013). Forced expression of *PKM2* isoform in cultured myotubes lowers the oxygen consumption rate and increases the glucose consumption rate. Furthermore, morpholino-based promotion of *PKM2* splice isoform in mice results in increased energy expenditure (Gao and Cooper, 2013). Taken together, these data suggest that missplicing of *PKM* disrupts energy metabolism in DM1 skeletal muscle.

### Missplicing of TNNT2

Although the exact function of different splice isoforms of cardiac troponin T (*TNNT2*) is not understood, myofibrils expressing the isoform containing exon 5 are more sensitive to calcium when compared to the exon 5 skipped isoform (McAuliffe et al., 1990; Godt et al., 1993). Individuals with DM1 show increased inclusion of exon 5, which is antagonistically regulated by CELF1 and MBNL1 (Philips et al., 1998; Ho et al., 2004). Direct binding of CELF1 to the five CUG repeats in the *TNNT2* intron promotes the alternative exon inclusion whereas MBNL1 binding to the helical region of the stem-loop represses it (Philips et al., 1998; Mankodi et al., 2005; Warf and Berglund, 2007). The stem-loop in *TNNT2* pre-mRNA contains two mismatches, which resembles the structure of CUG repeats in *DMPK* 3'-UTR confirming MBNL1 prefers binding to the structured stem-loops with pyrimidine mismatches (Mooers et al., 2005; Warf and Berglund, 2007). Furthermore, exon 5 skipping is developmentally regulated and induced expression of CUG<sup>exp</sup> RNA or MBNL1 deletion in adult mouse heart shifts *Tnnt2* splicing pattern to the embryonic stage (Wang et al., 2007; Kalsotra et al., 2008).

## Pathology Beyond Splicing

Sequestration of MBNL1 accounts for the majority of splicing perturbations; yet, many aspects of DM1 cannot be explained by the splicing defects alone. For instance, when global effects of CUG repeat expression in muscle were compared, up to one-third

of the steady-state transcript level changes observed in repeat expressing HSA<sup>LR</sup> mice were completely absent in *Mbnl1*<sup>Δ3/Δ3</sup> mice (Osborne et al., 2009; Du et al., 2010). Many of these changes occur in the genes involved in extracellular matrix function and some are implicated in other muscular dystrophies and connective tissue diseases. While direct evidence for what drives these transcript level changes is missing, several attractive hypotheses exist including depletion of other MBNL paralogues and increase in CELF1 activity. CUG<sup>exp</sup> RNA also disrupts other cellular processes including “leaching” of transcription factors (Ebraldize et al., 2004), abnormal DNA methylation (Lopez Castel et al., 2011), processing of repeats into small RNAs that produce unwanted gene silencing (Krol et al., 2007), activation of PKC-dependent signaling pathways (Kuyumcu-Martinez et al., 2007), bidirectional transcription (Nakamori et al., 2011), repeat associated non-ATG translation (Zu et al., 2011), microRNA deregulation (Perbellini et al., 2011; Rau et al., 2011; Fernandez-Costa et al., 2013; Kalsotra et al., 2014), and nonsense-mediated decay pathway (Garcia et al., 2014). Some of these splicing-independent abnormalities are discussed below.

### RAN Translation

Termed as Repeat Associated Non-ATG Translation (RAN translation), the phenomenon was first discovered in spinocerebellar ataxia type 8 (SCA8) (Zu et al., 2011), a neurodegenerative disease caused by expansion of CTG•CAG repeats in the *ATXN8* gene (Koob et al., 1999). Cells that were transfected with an *ATXN8* minigene containing the CAG expansion but without a canonical ATG initiation codon unexpectedly produced homopolymeric PolyGln, PolyAla, and PolySer proteins in all three frames (Zu et al., 2011). These homopolymeric peptides formed aggregates both in the nucleus and cytoplasm. Because *DMPK* is transcribed bidirectionally (Nakamori et al., 2011), CUG expansions in DM1 also undergo RAN translation producing a DM1-PolyGln expansion protein in DM1 patients and mice (Zu et al., 2011). Remarkably, RAN translation efficiency increases proportionately with repeat tract size, and RAN translation occurs predominantly when the RNA forms hairpin structures (Cleary and Ranum, 2013). Furthermore, cells producing RAN protein products have increased annexin-V staining, suggesting that RAN products can induce apoptosis. How and whether RAN proteins directly affect DM1 pathogenesis will further improve our understanding of the molecular mechanisms that are currently focused more on the RNA toxicity.

### MicroRNA Deregulation

Tissue-specific changes in microRNA expression may also play a major role in DM1 pathology. For instance, it was recently demonstrated that nuclear accumulation of CUG<sup>exp</sup> RNA in mouse heart triggers a decrease in expression of a group of microRNAs including *miR-1*, which is normally upregulated during postnatal development (Kalsotra et al., 2014). The same subset is also downregulated in hearts of DM1 individuals, including several whose depletion is associated with cardiac arrhythmias and fibrosis. In a separate study, MBNL1 was shown to bind to *pre-miR-1*, which blocks the binding of LIN28 under normal conditions, a protein known to repress *miR-1* biogenesis (Rau et al., 2011). It was proposed that due to MBNL1 sequestration, LIN28 could readily bind and inhibit *miR-1* processing in DM1 cardiac tissues.

However, because *miR-1* and most other microRNAs affected in DM1 are unchanged in *Mbnl1*<sup>Δ3/Δ3</sup> mice and the fact that their primary microRNA transcripts are reduced in parallel, their downregulation in DM1 is likely due to transcriptional defects. Indeed, subsets of these microRNAs are direct targets of MEF2 family of transcription factors, which are reduced in hearts of DM1 patients and mice (Kalsotra et al., 2014). Consequently, loss of MEF2 activity in DM1 heart tissue perturbs the MEF2-microRNA regulatory circuit resulting in global reprogramming of the cardiac transcriptome.

In DM1 skeletal muscle, alterations in microRNA expression have been reported both at the steady state levels and in their cellular distribution (Gambardella et al., 2010; Perbellini et al., 2011). Comparison of microRNA expression in a DM1 drosophila model and muscle biopsies of DM1 patients showed that multiple conserved microRNAs including *miR-1*, *miR-7*, and *miR-10* are downregulated (Fernandez-Costa et al., 2013). As expected, several of the downstream targets of these microRNAs are upregulated in DM1 skeletal muscle. More recently, a set of nine microRNAs was identified that are deregulated in peripheral blood plasma of DM1 patients (Perfetti et al., 2014). *miR-133a* in particular displayed an inverse correlation with skeletal muscle strength. It remains to be seen if these circulating microRNAs can serve as non-invasive diagnostic biomarkers.

### Misregulation of Transcription Factors

Besides MEF2, a few other transcriptional networks may also be affected in DM1. Termed “RNA leaching,” transcription factors were redistributed from chromatin to ribonuclear foci upon CUG repeat RNA expression in a DM1 cell line (Ebraldize et al., 2004). The sequestration of SP1 for instance may contribute to the DM1 myotonia phenotype in addition to the non-sense-mediated decay of misspliced of *CLCN1*. Interestingly, the expression of the sequestered transcription factors are reduced as well, further increasing the impact of the RNA leaching phenomena.

MyoD is a key transcription factor for myogenic genes, and thus, crucial for differentiation of myoblasts to myotubes. CUG repeat expression in C2C12 cell culture model reduces MyoD levels (Amack et al., 2002). Since MyoD transcript levels, transcript length, and localization are not affected in DM1 cells, the reduction in MyoD is possibly due to changes in its translational efficiency or protein stability. Nonetheless, reduced expression of MyoD may result in impaired differentiation capabilities due to aberrant expression of its target genes. However, this conclusion was contradicted in a more recent study, which demonstrated that not all DM1 patient cell lines exhibit impaired myogenesis (Loro et al., 2010). Instead, the observed atrophy of cultured DM1 myotubes was proposed to be a result of apoptosis and autophagy.

The cardiac transcription factor NKX2-5 regulates the expression of genes that are important for normal cardiac conduction. NKX2-5 is overexpressed in heart and skeletal muscle of DM1 patients (Yadava et al., 2008), which is recapitulated in mice overexpressing the *DMPK* 3'-UTR containing CUG repeats. NKX2-5 expression correlates with muscle pathology severity in both DM1 patient samples and DM1 mouse model (Gladman et al., 2014). Overexpression of *Nkr2-5* in C2C12 culture inhibits myogenic differentiation, while overexpression of *Nkr2-5* in mice exhibits decreased muscle strength, worsened muscle histopathology, and impaired muscle regeneration. Downstream

targets of Nkx2-5, such as *Nppb* and *Ankrd1*, were significantly up regulated in these mice upon transgene expression (Yadava et al., 2008). Moreover, *Nkx2-5* haploinsufficiency had a cardioprotective effect on the toxic RNA-expressing mice, which suggests NKX2-5 is a modifier of RNA toxicity. Whether misregulation of transcription factors such as MEF2, MyoD, and NKX2-5 is a primary event in response to CUG<sup>exp</sup> RNA expression and what mechanisms affect their normal expression in DM1 remain to be determined.

### Mechanisms That Reduce RNA Toxicity

There is mounting evidence for the role of cellular defensive mechanisms in reducing toxic RNA expression in DM1. The nonsense-mediated decay (NMD) pathway is a surveillance mechanism that detects mRNA with premature stop codons and targets them for degradation to prevent the expression of potentially toxic truncated proteins (Chang et al., 2007). RNAi silencing of smg-2, an RNA helicase component of the NMD pathway, in a DM1 *C. elegans* model causes an increase in CUG repeat containing mRNA (Garcia et al., 2014). Mutations in other NMD pathway genes, specifically smg-1 and smg-6, also resulted in a similar increase in CUG repeat RNA. Addition of non-repeat GC-rich elements into the 3'-UTR of a GFP reporter was sufficient to reduce GFP expression, whereas mutations in smg-1, smg2, or smg6, elevated the GFP expression. Thus, the NMD pathway plays a direct role in reducing toxic RNA expression through recognition of GC-rich elements. This recognition is nucleotide composition specific but not sequence specific, suggesting that the secondary structure formed by these GC-rich regions may be important for NMD recognition.

Dicer is a ribonuclease that cleaves double-stranded RNA to produce 21 nucleotide duplexes, which act as guides for the RNA-induced silencing complex (RISC) to target mRNAs for degradation (Bernstein et al., 2001; Elbashir et al., 2001). CNG (N=A, C, U, or G) repeat RNA can also act as Dicer substrate, with Dicer exhibiting higher affinity for CAG and CUG repeats (Krol et al., 2007). Interestingly, DM1 patient-derived cells showed a 40% decrease in mutant *DMPK* transcript (containing 1,000 CUG) compared to DM1 patient cells treated with siDicer, demonstrating a possible role for Dicer in reducing RNA toxicity. Another double stranded RNA-binding protein, Staufen-1, is increased in DM1 patient skeletal muscles and mouse models (Ravel-Chapuis et al., 2012). Staufen1 interacts with CUG<sup>exp</sup> mRNA, aids in its nuclear export and translation, and surprisingly rescues alternative splicing of two key pre-mRNAs that are aberrantly spliced in DM1.

Overall, these studies suggest that DM1 pathogenic mechanisms are complex and comprise changes in alternative splicing and gene expression, unconventional translation, and microRNA deregulation.

### From Mechanism to Therapeutics

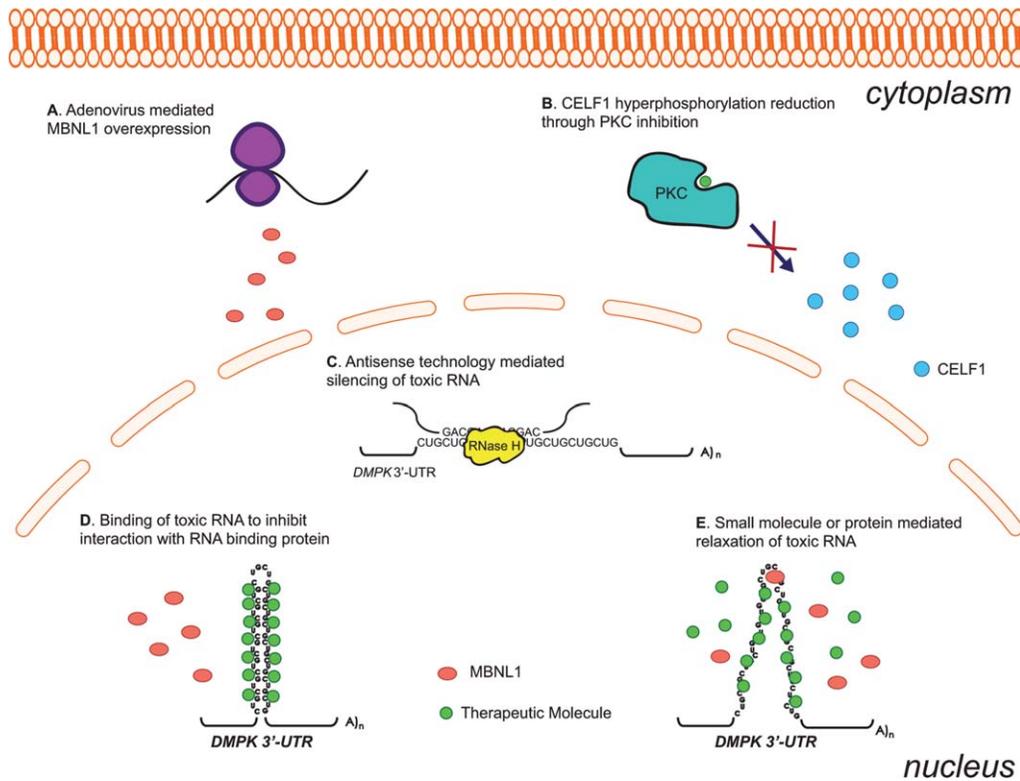
There are no treatments for DM1 at this time, and drugs currently in clinical trials are only for symptom management. However, the currently available data describing the major pathological mechanisms allows for targeted therapeutic development. MBNL1 loss of function accounts for >80% of splicing pathology in mice models, so overexpression of MBNL1 through virus infection could be one viable therapeutic approach (Fig. 3A). This is sup-

ported by previous studies that demonstrated overexpression of MBNL1 ameliorates splicing abnormalities, myotonia, and myopathy in DM1 mice (Kanadia et al., 2006; Chamberlain and Ranum, 2012). Reducing CELF1 steady state levels could combat some of the DM1 symptoms as well. Inhibition of the PKC pathway to reduce CELF1 phosphorylation and steady state levels increases survival rate in mice (Wang et al., 2009) (Fig. 3B), while *Celf1* knockout mice expressing the *DMPK* 3'-UTR-(CUG)<sub>5</sub> transcript are more resilient to muscular pathology (Kim et al., 2014). Combination therapy modulating MBNL1 and CELF1 levels could also be an effective approach to address DM1 symptoms, but direct targeting of RNA-binding proteins could have deleterious effects (Childs-Disney et al., 2013).

Since DM1 results from the formation of toxic gain of function RNA, targeting the toxic RNA should provide the most comprehensive results. Targeting of the toxic RNA can be approached in two ways, either targeting it for degradation, or covering the RNA in order to render its binding sites unavailable for interaction with RNA-binding proteins. One such approach to target the toxic repeat RNA is antisense oligonucleotides (ASO), which has been the most successful therapeutic development so far (Fig. 3C). One of the first applications of ASOs towards DM1 was the use of a retrovirus that expressed an ASO complementary to (CUG)<sub>13</sub> and the preceding 110 bp (Furling et al., 2003). DM1 myoblasts expressing the ASO showed marked improvement in myotube formation, fusion index, and glucose uptake. CELF1 expression was reduced to wildtype levels as well. However, ASO expression reduced both repeat containing and normal *DMPK* transcripts, so further modifications are needed to increase specificity towards the mutant transcript.

The development of CAG7, a fully 2'-O-methyl-phosphorothioate modified ASO complementary to CUG repeats, provided proof of principle that ASO-mediated silencing can be applicable for DM1 treatment to correct missplicing and reduce ribonuclear foci formation in vivo (Mulders et al., 2009). This was shortly followed by the development of CAG25, which was the first demonstration of morpholino ASO application for DM1 (Wheeler et al., 2009). Morpholino ASO binds to the target RNA and sterically inhibits interaction with the RNA-binding proteins. Treatment of HSA<sup>LR</sup> mice with CAG25 resulted in a significant reduction of RNA foci, reduction of CUG<sup>exp</sup> RNA, re-distribution of MBNL1, rescue of missplicing, reduction of myotonia, as well as increased translation of the CUG-repeat mRNA. Since morpholino oligonucleotides do not induce cleavage of target mRNA, CAG25 should not cause degradation of endogenous CUG-repeat carrying transcripts.

ASOs containing 8–10 consecutive phosphorothioate nucleotides at the center for RNase H binding were developed in order to test the efficacy of ASOs that are capable of eliciting RNase H-mediated degradation (Lee et al., 2012). Termed “MOE-gapmers” these ASOs are additionally flanked by 2'-O-Methoxyethyl modified nucleotides for increased nuclease resistance and RNA affinity. Treatment with MOE-CAG14 gapmer significantly reduced ribonuclear foci formation in vivo, but reversal of splicing abnormalities was only modest. The lack of improvement in splicing could be due to an insufficient decrease in ribonuclear foci, or a switch towards embryonic splicing pattern triggered by muscle regeneration in response to muscle damage. Both MOE-CAG14 gapmer and MOE-CTG14 control gapmer treatment induced muscle regeneration, indicating that gapmer treatments targeting a repeat sequence may induce muscle damage.



**Fig. 3.** Schematic model of the possible therapeutic targets for DM1. **A:** Adenovirus-mediated MBNL1 overexpression can compensate for sequestered MBNL1. **B:** PKC inhibition can reduce hyperphosphorylation of CELF1 to decrease CELF1 steady state levels. **C:** Toxic RNA can be silenced by RISC via siRNA targeting, or through antisense oligonucleotides (ASOs) capable of eliciting RNase H mediated degradation. **D:** Toxic RNA can be targeted for binding to eliminate interaction with RNA-binding proteins using small molecules or ASOs. **E:** Toxic RNA can be targeted by proteins or small molecules to induce relaxation of secondary structure to release sequestered MBNL1.

Although ASO studies thus far have produced promising results, each study revealed issues that limited the efficacy of the treatment. Morpholino oligonucleotides are RNase H inactive, so treatment would require repeated, locally administered injections. MOE-CAG gapmer treatment induced muscle damage that may be due to the targeting of a repeat sequence. To circumvent the requirement of repeated injections, a modified hU7-snrRNA with 15 CAG repeats was developed for long-term reduction of toxic repeat RNA (Francois et al., 2011). In addition, design of ASO gapmers that targeted the 5'- or 3'-UTRs of the CUG repeat containing transcript in HSA<sup>LR</sup> mice demonstrated that targeting outside of the repeat tract can reduce toxicity and increase the viability of systemic delivery via I.P. injection (Wheeler et al., 2012). The effect of ASO gapmer targeting the 3'-UTR downstream of the repeat tract persisted strongly even after treatment had been discontinued for 31 weeks. Most importantly, nuclear retained CUG<sup>exp</sup> RNA was highly sensitive to RNase H mediated degradation, which can be taken advantage of in future ASO developments.

The first in vivo study of synthetic siRNA targeting CUG<sup>exp</sup> RNA produced the best results compared to all ASO or small molecule studies to date (Sobczak et al., 2013). siRNA treatment in HSA<sup>LR</sup> mice drastically reduced CUG repeat transcript levels and nuclear foci formation when observed 7 days after a single injection. Both siCAG and siCAG-tail (containing an additional 5'-AGUGUU-3' nuclear localization signal) treated mice exhibited almost complete reversion of aberrantly spliced genes and myotonia. A concern regarding siCAG and siCAG-tail treatment is

that several endogenous CUG-repeat containing transcripts were reduced as well. Further modifications would be required to reduce possible off target effects.

Despite the promising results from ASO development, small molecules continue to be the more attractive therapeutic approach due to their efficiency and low cost of production as well as simplicity of administration. Small molecule-based strategies generally involve binding of the CUG<sup>exp</sup> RNA to sterically inhibit interaction with RNA-binding proteins (Fig. 3D). The first small molecule to demonstrate efficacy in DM1 cell culture models was pentamidine, which was discovered in a screen of nucleic acid binding compounds for disruption of MBNL1 binding to CUG<sup>exp</sup> RNA (Warf et al., 2009). Pentamidine treatment fully rescued splicing defects in a DM1 cell culture model. Although its ability to rescue splicing defects in HSA<sup>LR</sup> mice was modest at best, this study was the first to demonstrate that small molecules are a viable therapeutic approach for DM1.

The second significant step in the search for small molecule therapeutics occurred with the evidence that modular assembly methods and chemical similarity searching could be applied to improve the specificity and affinity of previously discovered RNA-binding compounds. Using an azide handle to modularly assemble a Hoechst 33258 [a known RNA 5'CUG/3'GUC binding molecule (Cho and Rando, 2000)] derivative on a peptoid backbone yielded several compounds that exhibit more significant improvement in potency, selectivity, cell permeability, and localization than the parent compound (Pushchnikov et al., 2009; Childs-Disney et al., 2012). This proved that multivalent

compounds could be modularly assembled to take advantage of the numerous binding sites present on CUG<sup>exp</sup> hairpin RNA for improved potency. Furthermore, a comprehensive study on the effect of backbone composition in modularly assembled compounds demonstrated that peptide tertiary amides (PTA) scaffolds provide improved bioactivity over polyamine,  $\alpha$ -peptide, or  $\beta$ -peptide scaffolds (Rzuczek et al., 2013). Chemical similarity searching with pentamidine and Hoechst 33258 as query molecules, using the National Cancer Institute and eMolecules database, provided compounds with increased efficacy for improving DM1 splicing defects in vivo compared to the parent compounds (Parkesh et al., 2012). Taken together, these studies indicate that not only is screening for hit compounds capable of binding DM1 repeat RNA a viable therapeutic approach, but modular assembly of multivalent compounds can drastically improve the efficacy of hit compounds.

The third significant step in small molecule therapeutics development was the discovery of heptamidine, which demonstrated for the first time that small molecule therapeutics can revert DM1 phenotype in an animal model (Coonrod et al., 2013). Heptamidine was discovered through experimentation with the methylene linker length in pentamidine to produce additional analogues. Heptamidine, which has a 7-carbon linker, significantly reduced myotonia and rescued missplicing of *Clcn1* and *Atp2a1* in HSA<sup>LR</sup> mice.

In addition to pentamidine and Hoechst 33258 derived compounds, a triaminotriazine-acridine conjugate has been discovered to inhibit MBNL1-CUG repeat complex formation (Arambula et al., 2009). However, the compound was ineffective in cell culture due to its low solubility and low permeability. To improve upon these parameters, a cationic polyamine side chain was attached to take advantage of the polyamine transporting system of cells (Jahromi et al., 2013b). The resulting compound exhibited partial rescue of *IR* minigene missplicing. In order to further improve upon the triaminotriazine-acridine hit compound, a small library of bivalent compounds containing two triaminotriazine-acridine units linked by various linker types and lengths was synthesized (Jahromi et al., 2013a). Compound 9, which contained a polyamine linker, exhibited a 266-fold increase in potency compared to the monomer. Despite the efficacy of the triaminotriazine-acridine compounds in dispersing RNA foci in cell culture, toxicity remained a significant problem. Upon realization of the fact that the CUG repeat hairpin structure was very similar to the HIV-1 frameshift RNA stem loop, a new compound was synthesized based on DB213, a small molecule binder of the HIV-1 frameshift RNA stem loop (Marcheschi et al., 2011; Wong et al., 2014). This compound, which replaced the dimethylammonium groups with triaminotriazine units, exhibited reduced toxicity and was able to improve the glossy and rough eye phenotype in a *Drosophila* DM1 model.

In addition to ASOs and small molecules, other therapeutic approaches show some promise as well. The discovery of ABP1 peptide is one such example (Garcia-Lopez et al., 2011). This peptide was discovered in a screen of D-amino acid hexapeptides using the *Drosophila* model. ABP1 was able to induce relaxation of CUG repeat hairpin structure in vitro, and could bind the CUG<sup>exp</sup> RNA without displacing MBNL1 (Fig. 3E). In addition, ABP1 treatment in HSA<sup>LR</sup> mice improved muscle histopathology and partially rescued missplicing of several genes. The most important advantage of this peptide is that it directly targets the CUG repeat hairpins and induces the relaxation of its secondary

structure, which indirectly prevents the binding of MBNL1 to the RNA. This mechanism of action would not affect endogenous transcripts containing short CUG repeats, a significant issue among the ASOs. Future screening of peptide libraries could yield additional hits, especially with the development of more precise screening methods based on ribonuclear foci elimination and high content imaging in DM1 cells (Ketley et al., 2014).

## Future Directions

Much of the molecular basis of DM1 pathology has been successfully uncovered in the past decade. The investigation into DM1 has led to many unexpected discoveries that have increased our understanding and appreciation of the nature of repeat expansion diseases. Although the pathogenic mechanism has only become increasingly more complex as more details are uncovered, the currently available information and animal models serve as excellent tools for further disease characterization and efficient development of potential therapeutics that can address the most debilitating symptoms. Progress towards new therapeutics has been accelerating since the discovery of Hoechst33528 in 2000, with a significant influx of studies published within the last five years. ASOs have demonstrated promising results in mice models, while improvements in screening and rational design techniques have accelerated development for increasingly potent small molecules. At the current rate of progress, new therapeutics could be available in the near future. For instance, ISIS Pharmaceuticals recently began phase 1 clinical trials for an antisense-based therapeutic designed to target mutant *DMPK* RNA, named ISIS-DMPKRx

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