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Compr Physiol. Author manuscript; available in PMC 2024 August 14.

Published in final edited form as:

Compr Physiol. ; 8(2): 509–553. doi:10.1002/cphy.c170002.

Myotonic Dystrophy and Developmental Regulation of RNA Processing

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Abstract

Myotonic dystrophy (DM) is a multisystemic disorder caused by microsatellite expansion mutations in two unrelated genes leading to similar, yet distinct, diseases. DM disease presentation is highly variable and distinguished by differences in age-of-onset and symptom severity. In the most severe form, DM presents with congenital onset and profound developmental defects. At the molecular level, DM pathogenesis is characterized by a toxic RNA gain-of-function mechanism that involves the transcription of noncoding microsatellite expansions. These mutant RNAs disrupt key cellular pathways, including RNA processing, localization, and translation. In DM, these toxic RNA effects are predominantly mediated through the modulation of the muscleblind-like and CUGBP and ETR-3-like factor families of RNA binding proteins (RBPs). Dysfunction of these RBPs results in widespread RNA processing defects culminating in the expression of developmentally inappropriate protein isoforms in adult tissues. The tissue that is the focus of this review, skeletal muscle, is particularly sensitive to mutant RNA-responsive perturbations, as patients display a variety of developmental, structural, and functional defects in muscle. Here, we provide a comprehensive overview of DM1 and DM2 clinical presentation and pathology as well as the underlying cellular and molecular defects associated with DM disease onset and progression. Additionally, fundamental aspects of skeletal muscle development altered in DM are highlighted together with ongoing and potential therapeutic avenues to treat this muscular dystrophy.

Introduction

Myotonic dystrophy (*dystrophia myotonica*, DM) is a dominantly inherited and highly variable disease that affects nearly every organ system in the body (154). There are two types of DM defined by genetic etiology. DM type 1 (DM1) is caused by a CTG expansion (CTG^{exp}) in the 3’ untranslated region (UTR) of *dystrophia myotonica protein kinase* (*DMPK*), while a CCTG expansion ($CCTG^{exp}$) in the first intron of *cellular nucleic acid binding protein* (*CNBP*) leads to DM type 2 (DM2) (47, 231). In contrast to DM2, DM1 also occurs as a congenital disease (CDM) due to maternal transmission of exceptionally large (typically > 1000) CTG^{exp} *DMPK* mutations (257). In fact, a variety of DM clinical symptoms are distinguished by their age-of-onset, systems involvement, and presentation

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(257). Skeletal muscle involvement is one of the most striking clinical manifestations of DM patients. Patients present with myotonia (delay in muscle relaxation following contraction), weakness of limb and facial musculature, and progressive adult-onset muscle wasting. Underscoring the dramatic variability of this disease, skeletal muscle development defects, including hypotonia (low basal muscle tone), are a characteristic neonatal feature of CDM together with perinatal mortality associated with respiratory insufficiency and swallowing difficulties. Cardiac and neurological dysfunctions are other prominent features of DM and contribute to patient mortality and diminished quality of life, respectively.

The molecular basis of DM pathogenesis has been the subject of intense investigation since the discoveries of the DM1 and DM2 mutations. Today, the prevailing pathomechanism is transcription across C(C)TG^{exp} tracts produces toxic C(C)UG^{exp} RNAs that disrupt the normal functions of effector proteins, most notably members of the muscleblind-like (MBNL) and CUGBP and ETR-3-like factor (CELF) families of RNA binding proteins (RBPs) (135, 319, 359). MBNL and CELF proteins are involved in diverse RNA processing steps, including alternative splicing (AS), alternative cleavage and polyadenylation (APA), mRNA stability, RNA localization, mRNA translation, and microRNA (miRNA) biogenesis (26, 203, 322, 428, 429). Interrogation of these MBNL- and CELF-responsive activities, and their misregulation in DM, has elucidated links between specific RNA processing events and patient symptoms and enhanced our understanding of the roles of certain RBPs in the developmental regulation of RNA processing. Current efforts are focused on comprehensive surveys to characterize the extent of RNA misprocessing events within the DM transcriptome. Many of the cellular pathways highlighted in these studies have known roles in skeletal muscle development and maintenance, and a common theme is the retention of developmentally immature RNA processing patterns in adult tissues.

In this review, we provide a comprehensive survey of DM skeletal muscle pathophysiology and highlight seminal studies that led to relatively rapid progress from the identification of the causative mutations to the development, and current implementation, of rationally designed therapeutics. We begin with a brief historical overview of DM research followed by a discussion of the clinical and histological hallmarks of DM skeletal muscle. Next, we examine working hypotheses and molecular models of DM pathogenesis leading to skeletal muscle dysfunctions that have been garnered through *in vitro*, *in vivo*, and *in silico* analyses. Finally, we summarize current and potential therapeutic interventions and conclude by addressing emerging and largely unresolved questions remaining in the DM field.

Historical Perspective

Disease characterization to causative mutation

In 1909, Hans Steinert described patients presenting with myotonia (Fig. 1) and progressive muscle wasting coupled with multisystem involvement, which led to the initial designation of this disorder as Steinert’s disease (332,381). Despite the autosomal dominant inheritance pattern, the variability of Steinert’s disease obscured its genetic etiology for decades. This was due in part because, unlike disorders associated with direct links between protein loss-of-function and disruption of tissue homeostasis (e.g., *dystrophin/DMD* mutations and Duchenne muscular dystrophy or DMD), consolidating the molecular mechanisms

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governing the pleiotropy of Steinert's disease was, and remains, a complex task. Furthermore, the observation of increased symptom severity and decreased age-of-onset in successive generations of affected families, or genetic anticipation, underscored the complex and variable nature of this disease. Once dismissed as ascertainment bias, the molecular mechanism underlying anticipation was later identified following the sequencing of the disease-linked *DMPK* mutant gene (117, 150, 151). Steinert's disease is one of the most striking examples of anticipation. For example, a mutation-harboring grandparent may be largely asymptomatic while her daughter presents with adult-onset disease that is diagnosed following the birth of her severely affected congenital infant, the proband. Indeed, the birth of a child with CDM is a common impetus for evaluation of affected families (251, 399). As studies of Steinert's disease continued throughout the 20th century, the disorder was eventually renamed myotonic dystrophy, or DM, after its hallmark muscle symptoms.

The genetic basis of this classical form of DM was revealed in 1992, with the identification of a CTG^{exp} in the 3' UTR of *DMPK* located on chromosome 19q13.3 (Fig. 2) (47, 55, 150, 241). While unaffected individuals possess between 5 and 37 *DMPK* CTG repeats, disease symptoms emerge when the CTG^{exp} surpasses 50 repeats. Thus, DM became the third disease associated with unstable nucleotide expansions after fragile X syndrome (FXS) and X-linked spinal-bulbar muscular atrophy (or Kennedy's disease). Beyond a disease-specific threshold, these repeat tracts are unstable and prone to intergenerational and somatic expansions. Importantly, the discovery of microsatellite expansions provided a mechanistic foundation for understanding genetic anticipation that is associated with multiple microsatellite expansion disorders including FXS, Huntington disease (HD), and several types of spinocerebellar ataxia (SCA) (117, 206, 212). In DM, the earlier age-of-onset and increased severity of symptoms generally correlates with CTG^{exp} repeat number (Fig. 3). The repeat expansions associated with clinically defined DM manifestations range from mild/asymptomatic (~50–<150), classic (~50–<1000) to congenital (>1000) (Fig. 4) (257).

Following the identification of the *DMPK*-linked CTG^{exp} mutation, reports emerged of patients presenting with DM-like symptoms who tested within the normal CTG^{exp} range and showed preferential proximal, rather than distal, muscle involvement (331, 400). This disorder was originally termed proximal myotonic myopathy (PROMM). In the late 1990s, the casual mutation was linked to chromosomal region 3q21 and later revealed to be a $CCTG^{exp}$ in the first intron of *CNBP* (originally termed *zinc finger 9, ZNF9*) (Fig. 2) (231, 321). Shortly afterward, the *DMPK*-linked disease was renamed DM type 1 (DM1) and PROMM was designated DM type 2 (DM2) based on their phenotypic similarity, yet distinct etiology and presentation. In the context of DM2, normal individuals have < 30 CCTG repeats while disease manifestations have been observed in patients with as few as 55 $CCTG^{exp}$ repeats (231). In general, DM2 is later onset and less severe compared to DM1 and a congenital form of DM2 has not been reported (Fig. 4). Additionally, the prevalence of DM1 (1 in ~8000) is greater and more widespread than DM2 (~3% of DM cases worldwide) except in some regions of Northern Europe where the epidemiology is more comparable (254, 415). However, underdiagnosis of DM2 may be prevalent since it is often a late-onset disease and therefore confounded by the normal aging process (320).

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Mutations to disease models

DM-associated C(C)TG^{exp} mutations are located outside conventional protein-coding regions of the genome (Fig. 2), leading to a fundamental question: how do DNA simple sequence repeats in noncoding regions result in disease? Both forms of DM are inherited in an autosomal dominant pattern and *DMPK* and *CNBP* missense mutations have not been linked to either DM1 or DM2 arguing against a loss-of-function model. The similarity between DM1 and DM2 suggested shared pathogenic mechanisms might exist.

Two initial models emerged to reconcile these observations. Reduced expression of mutation harboring (*DMPK*, *CNBP*), and/or flanking (*SIX5*, *DMWD* for DM1), genes could result in haploinsufficiency. Alternatively, or in addition to haploinsufficiency, transcription across C(C)TG^{exp} regions could generate toxic gain-of-function RNAs. The haploinsufficiency model garnered initial support since reduced *DMPK* mRNA and protein levels were reported in adult DM1 tissues (120). However, these results remain controversial, as other groups reported decreased, similar, or increased levels of *DMPK* mRNA and protein (59, 340, 432). Discrepancies in reported *DMPK* mRNA levels are likely due to the methodologies utilized for RNA isolation and analysis. While transcripts from the normal *DMPK* allele are readily purified using phenol-chloroform-based extraction procedures, expanded transcripts remain insoluble and are lost during purification (85, 147). The use of cesium chloride gradient-based purification techniques circumvented this issue and revealed normal steady-state levels of mutant *DMPK* transcripts (85). Nonetheless, CUG^{exp}-containing *DMPK* transcripts may be susceptible to reduced translation. Therefore, *Dmpk* knockout (KO) mice were generated to test the hypothesis that DMPK depletion contributes to disease (Table 1) (179, 327). While homozygous *Dmpk* KO animals display mild phenotypes, heterozygous *Dmpk* KOs, a true model of haploinsufficiency, do not recapitulate features of DM arguing against a substantial role for DMPK loss-of-function. Additionally, the DM2 mutation is in a different gene on another chromosome, which suggests that the haploinsufficiency model fails to explain aspects of pathogenesis common between DM1 and DM2.

As early as 1995, several hypotheses were proposed that DM1 was caused by a dominant-negative RNA gain-of-function mechanism (432). One study suggested that poly(A)$^{+}$ RNA accumulation was blocked *in trans* by the *DMPK* expansion allele transcript but this finding was later refuted (432). However, another observation was made while tracking mutant *DMPK* transcript distribution in patient-derived cells. Using RNA fluorescence *in situ* hybridization, mutant *DMPK* transcripts were observed as punctate aggregates, or RNA foci, in the nuclei of DM1 patient-derived cells and tissues (Fig. 5) (85, 391). While unexpanded transcripts are efficiently exported to the cytoplasm, mutant *DMPK* transcripts are almost completely retained in the nucleus (85, 147). Strikingly, intranuclear CCUG^{exp} RNA foci were also observed in DM2 patient-derived cells and tissues (Fig. 5) (231). These observations provided the first evidence for a shared mechanistic link between DM1 and DM2 and sparked considerable interest concerning the potential toxicity of these RNA aggregates.

The RNA gain-of-function model was also tested using mouse models (Table 1). Early attempts to study the effects of CTG^{exp} tracts *in vivo* utilized mice containing a DM1 *DMPK* transgene (265). While these animals showed a variety of phenotypes, it was unclear

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if the CUG^{exp} RNAs were sufficient to generate disease independent of the *DMPK* gene context. To test this possibility, a transgenic mouse model was generated that expressed either a CTG^{5} (*HSA*^{SR}) or a CTG^{250} (*HSA*^{LR}) expansion, inserted into the 3’ UTR of a human skeletal actin transgene (245). Only the *HSA*^{LR} transgenic model recapitulated aspects of DM-associated myopathy, including myotonia, centralized myonuclei, and nuclear RNA foci (Fig. 5). Importantly, the severity of these features correlated with the degree of transgene expression demonstrating that the repeat expansion was toxic at the RNA, or a downstream, level. This study provided the first conclusive evidence that CUG^{exp} RNAs could exert their toxic effect independent of gene context.

The remaining unresolved link for the RNA gain-of-function hypothesis was to determine the molecular pathways downstream of CUG^{exp} expression that are adversely affected in disease and how CUG^{exp} RNAs disrupt cellular homeostasis. Our group proposed a protein sequestration model in which CUG^{exp} RNAs recruited, and subsequently sequestered, cellular factors with a high affinity for expanded CUG repeats. As an initial step to characterize these factors, *in vitro* pull-down assays were performed with (CUG)^{8} and these studies led to the identification of CUG-binding protein 1, CUGBP1 (currently CELF1) (406). However, further characterization of CELF1 did not support the hypothesis that this protein is a sequestered factor: (1) CELF1 binding to CUG^{exp} RNA was not proportional to repeat number; (2) CELF1 failed to colocalize with CUG^{exp} RNAs in RNA foci; and (3) CELF1 steady-state levels were upregulated in patient-derived cells (307, 406). Thus, the question of whether sequestered factors existed remained unresolved. To address this concern, an alternative experimental approach was tested that involved UV-crosslinking of proteins to radiolabeled RNAs following *in vitro* RNA processing reactions in HeLa cell nuclear extracts. These studies identified proteins homologous to *Drosophila* muscleblind (Mbl) (263). Importantly, these human proteins, termed MBNL, bound to CUG^{exp} RNAs in a length-dependent manner, suggesting elevated sequestration as repeat tracts increase. Furthermore, all three human MBNL paralogs interact with CUG^{exp} and CCUG^{exp} RNAs *in vitro* and patient nuclei *in vivo* (105, 106, 247, 263, 386).

Another proposed, but not mutually exclusive, mechanism for C(C)UG^{exp} toxicity in DM is a noncanonical form of protein translation termed repeat associated non-ATG (RAN) translation, which was originally discovered during studies on SCA8 pathomechanisms (78,463). *DMPK*, similar to other genes affected by microsatellite repeat expansions, is bidirectionally transcribed (27) so sense CTG repeats have the potential to encode polyleucine (polyLeu), polyalanine (polyAla), and polycysteine (polyCys) while the antisense CAG strand would translate into polyglutamine (poly-Gln), polyAla, and polyserine (polySer). Of these potential RAN products, only polyGln has been observed in DM1 myoblasts and skeletal muscle although this polyGln accumulation is more prevalent in blood cells (463).

Theories to therapies

Since both MBNL and CELF proteins were implicated in DM pathogenesis, understanding their normal cellular functions was the next critical step into understanding the molecular events misregulated in disease. Early work on CELF1 demonstrated that it was an AS factor

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that regulated human cardiac troponin T (cTNT) pre-mRNA splicing (307). Subsequent studies demonstrated that missplicing of the muscle chloride channel, CLCN1, was also responsive to CELF1. This missplicing event leads to nonsense-mediated decay of CLCN1 mRNA, which results in the myotonia, or muscle hyperexcitability, observed in DM. Subsequently, MBNL proteins were also shown to regulate the AS of gene transcripts misregulated in DM1 and DM2 (164, 189). DM-relevant missplicing also occurs in both *HSA*^{LR} transgenic and *Mbnl1* KO mouse models and both animal models develop myotonia (189, 245). Currently, many RNA missplicing events have been identified in patient samples and animal models (Table 2) (67, 246, 272). Many of the affected pre-mRNAs (e.g., *CLCN1*, *TNNT3*, and *INSR*) are functionally related to known aspects of DM skeletal myopathy including myotonia, muscle weakness, and insulin insensitivity. A recurring theme from studying these splicing patterns is the retention of fetal exons in mature tissues and the antagonistic roles of MBNL and CELF proteins for some RNA targets (230). For example, *CLCN1* exon 7a inclusion, which is the predominant pattern in embryonic and neonatal tissue of the developing mouse, leads to transcript degradation and possibly production of a CLCN1 C-terminal truncated protein (Fig. 6) (67, 246). As the muscle matures, *CLCN1* exon 7a is increasingly excluded from the mRNA, facilitating increased *CLCN1* RNA stability and downstream translation. For this event, CELF1 and MBNL1 promote inclusion and exclusion, respectively, leading to the aberrant retention of *CLCN1* exon 7a in DM skeletal muscle when the activities of these RBPs are disrupted. In agreement with studies in the mouse, CLCN1 is lost from DM patient tissues and is associated with myotonia.

The advent of high-throughput transcript analysis technologies, such as microarrays and RNA-seq, have uncovered many RNA processing defects in DM. Furthermore, the use of crosslinking/immunoprecipitation and high-throughput sequencing (CLIP-seq) revealed direct MBNL and CELF binding targets, which has led to the identification of direct drivers of myopathy and other disease manifestations versus RNA processing errors resulting from generalized cellular dysfunction. Beyond a spliceopathy, DM is also a multifaceted RNA processing disorder as novel functions of MBNL and CELF proteins have emerged, including roles in alternative polyadenylation, RNA localization, miRNA biogenesis, RNA turnover, and control of translation. Today, unraveling the relative involvement of the complex mechanisms contributing to DM is an ongoing area of research. Identification of disease mediators is providing opportunities to design rationale therapeutics, such as antisense olignonucleotides (ASOs) targeting toxic RNA (438) or the use of pharmaceuticals to ameliorate symptoms (234).

DM Clinical Presentation

DM is a multisystemic muscular dystrophy affecting nearly every organ system of the body. A striking aspect of the DM1 phenotype results from skeletal muscle dysfunction, including myotonia, weakness, wasting, and myalgia (muscle pain) (Fig. 4) (332). However, DM is a truly multisystemic disorder with dysfunction of the cardiovascular system (arrhythmias, conduction blocks, cardiogenic syncope, and hypotension), respiratory system (respiratory muscle weakness, aspiration, and sleep apnea), gastrointestinal track (dysphagia, reflux, dyspepsia, choleostasis, constipation, diarrhea), central nervous system (hypersomnia, intellectual disability, executive dysfunction, peripheral neuropathy,

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and behavioral, emotional, and social difficulties), eye (particulate cataracts, ptosis, retinopathy, and ocular hypotension), endocrine system (insulin resistance/diabetes and metabolic syndrome), immune system (hypogammaglobulinemia), liver (steatosis and cirrhosis), reproductive system (testicular atrophy, female infertility, pregnancy, and neonatal complications), premature frontal balding, and pilomatrixoma (253,257,332,399). Many of these dysfunctions are more prominent in DM1 than DM2, and for overlapping features (e.g., myotonia), the severity is typically greatest in DM1. On the other hand, DM2 patients are more affected by myalgia (257).

Within DM1, subtypes are clinically defined based on age-of-onset and symptoms that correlate with repeat copy number: (1) late/asymptomatic; (2) adult/classic; (3) juvenile/childhood; and (4) congenital (257). In particular, CDM is often considered a unique disorder despite its shared genetic etiology to DM1 due to the exceptionally large CTG^{exp} alleles (>750–1000), prenatal onset, and the unique constellation of symptoms, which include reduced fetal movement, polyhydramnios, hypotonia, respiratory distress, talipes, hydrocephalus, arthrogryposis, and intellectual disability (399). There are no genetically or clinically defined DM2 subtypes.

The birth of a CDM infant is often the impetus for evaluation and diagnosis of DM1 affected families. In cases where individuals pursue an independent clinical evaluation, hypersomnia is typically the motivating complaint and often occurs prior to adult-onset myopathy. Interestingly, many patients are unaware of their myotonia prior to clinical evaluation. In all forms of DM, cataracts are an early prognostic event particularly for late-onset/asymptomatic cases of DM1. Molecular diagnosis has almost eliminated the need for diagnostic muscle biopsies and is the only definitive test to diagnose DM and distinguish between DM1- and DM2-linked mutations. While it is not clinically utilized for diagnosis, DM skeletal muscle histopathology is sufficiently characteristic to identify the disorder as DM and differentiate between DM1 and DM2 (discussed below). Due to the later onset and variably muted phenotype of DM2, there is underdiagnosis of affected individuals even for those carrying large $CCTG^{exp}$ mutations.

Hypersomnia and behavioral changes typically precede skeletal muscle involvement and are among the most substantial features affecting patient quality of life (213, 214). As progressive muscle weakness and wasting emerge, additional complications arise such as gait abnormalities and difficulties performing tasks requiring fine dexterity. Altogether, these dysfunctions can be physically and socially disabling, and many DM patients have trouble securing employment. Premature mortality, typically occurring within the fourth decade of life, is most frequently associated with cardiac dysfunction and/or severe skeletal wasting leading to respiratory insufficiency (420). Many aspects of DM1 skeletal muscle dysfunction similar to age-related sarcopenia, suggesting DM1 is a progeroid-like syndrome (242, 253). For DM2, myalgia is one of the primary complaints, but life span is not significantly reduced. While DM is a multisystemic disorder, we will focus on skeletal muscle involvement for the remainder of this review. In the current section, we will provide a clinical perspective of the core DM skeletal muscle manifestations (myotonia, weakness, and wasting) and a discussion of events unique to CDM skeletal myopathy.

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Myotonia

Following excitation of skeletal muscle by lower motor neurons, a variety of voltage-dependent, ion, and mechanosensory channels stimulate the release of calcium from extra- and intracellular stores. These events couple neuron-stimulated excitation to uniform and robust contraction of sarcomeres, a process termed excitation-contraction (EC) coupling. Following contraction, repolarization of the sarcolemmal membrane potential is required to allow stabilization of homeostatic calcium gradients and muscle relaxation. This process is also regulated by channel-mediated ion redistribution.

Myotonia is an abnormal delay in muscle relaxation following contraction, and occurs in the tongue, jaw, feet, and hand musculature of DM patients (25, 171, 434). While myotonia is widespread, it is initially assessed by grip myotonia—an abnormal delay in extending fingers after forming a fist (Fig. 1) (171). A more reliable diagnosis of myotonia is achieved by stimulating the thenar eminence (25). This is termed percussion myotonia and can be observed in the absence of grip myotonia (25). To obtain quantitative measurements of electrical myotonia, electromyography (EMG) is performed. Insertion of concentric needle electrodes elicits depolarization of the sarcolemma and results in measurable action potential number, duration, amplitude, and qualities (e.g., waxing and waning) (309). As electrical myotonia can be observed independent of grip and percussion myotonia, EMG is the most sensitive measurement of myotonia (18, 238, 434). Additionally, outputs from EMG can be useful in differentiating between myotonic versus other (e.g., inflammatory) myopathies (128, 160).

DM is one of several myotonic disorders including myotonia congenita, paramyotonia congenita, and hyperkalemic periodic paralysis (238,384). While these diseases result from mutations in specific ion channels, DM-associated myotonia results from aberrant *CLCN1* pre-mRNA splicing. As noted above, this missplicing destabilizes the transcript leading to its degradation and loss of CLCN1 from the sarcolemma. Because CLCN1 acts as a major regulator of chloride flux in mature muscle, its loss results in muscle hyperexcitability.

EMG-measured myotonic discharges are generally of greater frequency and amplitude in DM1 than DM2, and the distribution of myotonia differs in DM types with distal muscle groups more affected in DM1 while proximal muscle problems are more prominent in DM2 (160, 233). Additionally, DM2 action potentials are characterized almost exclusively by a waning characteristic (233, 452). Interestingly, in cases of pronounced myotonia in DM2 patients, there is often a cosegregating mutation in the *CLCN1* protein-coding region or *sodium channel, voltage gated, type IV alpha subunit* (*SCN4A*) (52, 385).

Chloride-mediated myotonic disorders, such as DM, display ameliorated severity following repeated contraction-relaxation episodes, known as the warm-up phenomenon (346). Consequently, patients have the greatest degree of difficulty initiating movement with improvements over time. DM patients are spared of sensitivity to cold climates, a feature frequently observed in sodium-mediated myotonic disorders (115, 128). Although the consequences of myotonia are most evident while performing tasks requiring fine motor skills, health may be compromised if severity reaches a debilitating threshold in bulbar muscles important for chewing and swallowing. Furthermore, myotonia in diaphragm

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and intercostal muscles may contribute to respiratory complications and life-threatening myotonia can be induced by the use of neuromuscular blockers during surgery. DM is one of the few dystrophic myotonic disorders, and as the disease progresses, myotonia typically becomes undetectable as muscle atrophy and weakness become more prevalent (378).

Muscle weakness

The contraction of muscle generates force, the magnitude of which is dictated by factors including myofiber number, size, and fiber type. Additionally, the efficiency of EC coupling and integrity of the contractile apparatus within a myofiber are critical components influencing muscle strength. Muscle is a highly plastic organ, and physical training can increase its size through hyperplastic and hypertrophic mechanisms culminating in a greater force potential. A balance of anabolic and catabolic pathways governs this plasticity with predominant catabolic activity contributing to muscle weakness in muscle disuse atrophy, aging, and disease. Furthermore, myofiber loss causes debilitating weakness. While these are examples of weakness subsequent to loss of myofiber size or number, defects in the contractile apparatus and/or architecture of the costamere also compromise muscle strength.

The assessment of muscle strength in a sensitive, quantitative, and reproducible manner is a nontrivial task. This is a particularly important consideration as clinic-to-clinic variability can lead to technical artifacts, hindering development of reliable data useful as clinical trial outcome measures. Hand-held dynamometry devices are a commonly utilized method to assess grip strength and meet the needs of quantitative output, reproducibility, and ease of use (377). However, for many muscle groups, the slow progressivity of weakness poses practical concerns for using traditional strength measurement scales (295, 441). The measurement of handgrip strength has been proposed as a possible exception, as changes can be detected within 6 months for most patients, a reasonable time scale to test therapeutic efficacy (441). These findings have been supported by a retrospective analysis of 204 DM1 patients, and both studies highlight the slow progressivity of weakness in the majority of DM muscle groups (45).

DM patients are affected by progressive muscle weakness with early signs of onset in the face, neck, ankles, and hands (238, 441). While this progressivity is slow, it can be debilitating in some instances (146, 279). Interestingly, weakness typically manifests secondary to myotonia, suggesting a temporal hierarchy in disease symptom emergence (238). Facial muscle weakness contributes to difficulties chewing and swallowing, ptosis, and the drooping appearance of the face. Weakness of the leg musculature impairs ambulation and is associated with difficulties lifting the foot, termed foot drop. Gait abnormalities and reduced muscle force in the legs correlates with increased propensity for falls in more severely affected DM patients (146). Late stage weakness in bulbar and respiratory (diaphragm and intercostal) muscles increase patient morbidity (238, 257).

As with myotonia, muscle weakness is more severe in DM1 than DM2, and DM1 shows a preferential involvement of distal rather than proximal muscles (257). Interestingly, DM1 is unique in the distal, rather than proximal, involvement seen in most myopathies including DM2, suggesting a particular sensitivity of muscles such as the *tibialis anterior* (TA) to CUG^{exp}-mediated toxicity (441).

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Muscle wasting

The normal development, function, and maintenance of muscle requires a complex interplay between transcriptional, co-/posttranscriptional, and downstream pathways (89). In general, a rigid network of extracellular support and a variety of membrane repair mechanisms help to maintain the structural integrity of muscle. Furthermore, resident skeletal muscle stem, or satellite, cells (SCs) provide muscle with a high regenerative capacity and also contribute to hypertrophy (98). In response to activity such as exercise, a variety of endocrine signals and mechanosensors stimulate growth receptors that activate anabolic signaling cascades, most notably the Akt/mTOR pathway (40). Conversely, muscle wasting, or atrophy, is the decline of muscle mass resulting from excessive catabolic activity and commonly occurs because of disuse, inadequate innervation, aging, and disease (39,80). Loss of muscle bulk can result from a decrease in individual myofiber size and/or a reduction in total myofiber number. Progressive muscle wasting leads to a dramatic decrease in body weight, muscle weakness, disability, and when severe, poses a formidable health risk. For example, cancer-induced muscle loss, or cachexia, is the proximal cause of death of many cancer patients (461).

Early signs of wasting in DM1 are seen in facial muscles (e.g., temporalis and masseter) and distal limbs. In late-stage disease, proximal muscles are similarly affected. DM2 patients experience preferential proximal muscle wasting and can unexpectedly present with hypertrophy of some distal muscles such as the gastrocnemius. While there is a scarcity of quantitative data regarding DM muscle wasting, qualitative assessment of patient muscle biopsies reveals features of degenerative disease and will be discussed in more detail below (423). Beyond myofiber wasting, satellite cell dysfunction may be compromised in DM (243, 244). While this has yet to be explicitly studied, the complex nature of muscle wasting in DM is likely to be explained by a combination of systemic pathology, defects inherent to myofiber function, increased proliferative burden of satellite cells, and ultimately exhaustion of the satellite cell population. As discussed below, many aspects of DM wasting resemble age-related sarcopenia (253).

Developmental abnormalities in CDM

While CDM is also caused by CTG^{exp} mutations in the *DMPK* 3' UTR, the exceptionally large repeat copy number (typically > 1000), unique constellation of symptoms, and prenatal onset is often the basis for its classification as a clinically distinct disorder (162, 256, 257). This is similar to the repeat-length-based criteria distinguishing fragile X-associated tremor/ataxia syndrome (FXTAS) and FXS (144).

Another critical distinction between DM1 and CDM is the fact that CDM patients inherit highly expanded alleles, resulting in the present of large CTG^{exp} mutations throughout embryogenesis. As such, several symptoms emerge *in utero* including reduced fetal movement, polyhydramnios, talipes, and borderline ventriculomegaly (399, 456). Polyhydramnios is likely reflective of myogenic defects impairing the ability of fetal swallowing (148, 355, 456). Perinatally, CDM infants present with hypotonia, poor suckling due to bilateral facial weakness, dysphagia, and respiratory insufficiency necessitating supportive ventilation (100). Hypotonia is one of the most visually striking feature of newborn CDM infants, and affects posture and movement leading to the hallmark "floppy

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baby" appearance (38, 301). Assisted feeding is necessary for the majority of CDM infants (57).

Diaphragm and intercostal muscle weakness contributes to respiratory insufficiency and is the greatest source of mortality (58, 326, 339). Perinatal asphyxia correlates with reduced APGAR score and measures of neurological function in later years of life, suggesting a contribution of muscle weakness to other phenotypes (390). CDM infants will show gradual improvement but display reduced motor milestones and eventually develop symptoms associated with childhood onset DM1 (100,399). Intellectual disability including autism spectrum disorder is a prominent feature (100,102). Although clinical and electrical myotonia eventually emerges, it is absent in CDM neonates likely due to the dispensable role of CLCN1 in immature muscle. Interestingly, many CDM symptoms are similar to those seen in other congenital myopathies. This observation suggests that different genetic defects affect developmental myogenesis to culminate in similar clinical presentations (175, 328).

Skeletal Muscle Architecture in DM

A skeletal muscle is organized into highly interconnected and organized structural units—an organizational pattern important for proper function (Fig. 7). Histological and ultrastructural examinations of diseased skeletal muscle offer insights into the degree of tissue and cellular pathology and even provides clues into the nature of molecular dysfunction. For example, disrupted transverse tubules morphology observed via electron microscopy is suggestive of defective EC coupling, calcium handling, and potential underlying dysfunction of dihydropyridine receptors.

An individual muscle is comprised of many muscle fascicles—bundles of myofibers surrounded by a connective tissue layer termed the perimysium. Within a single muscle fascicle are the muscle cells themselves, the myofibers, that contain the functional units of muscle contraction (sarcomeres) comprised of the thin (actin) and thick (myosin) filaments. Mature myofibers contain hundreds of myonuclei located beneath the sarcolemma. The positioning of organelles in a myofiber is important to avoid obstructing the sarcomere, thus allowing uniform and undisturbed contraction. While observation of typical muscle cross-sections reveals striking homogeneity, differences in fibers can be observed using a variety of staining methods to identify biochemical fiber types within a muscle, of which, there are four major categories—type I, IIA, IIB, and IIX—largely defined based on their relative sarcomeric myosin heavy chain composition (354). The relative proportion of these varies between muscle groups and dictates aspects of muscle performance. For example, TA is mostly a type II fast-twitch muscle, with a greater force potential than type I muscles. On the other hand, predominant type I slow-twitch muscles (e.g., *soleus*) are better suited for endurance tasks such as maintaining posture. Fiber-type patterning is first established during embryogenesis by homeoproteins such as SIX1 and SIX4, but has substantial postnatal plasticity based on hormones, innervation, electrical stimulation, and activity (141, 177, 330). Training regimes stimulate fiber-type transitions, typically in a predictable manner: I ↔ IIA ↔ IIX ↔ IIB (304). Type IIB fibers typically have the greatest cross-sectional area, providing the greatest force capacity. Fiber types also dramatically differ in their metabolic

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signature with type I being the most oxidative and type IIB the most glycolytic. Importantly, these differences provide the foundation for histochemical staining procedures allowing for the identification of different fiber types within a muscle cross-section (354).

DM1 and DM2 muscle structure profile

Haemotoxylin and eosin staining, a common technique for analysis of patient skeletal muscle biopsies, reveals several hallmark characteristics of DM histopathology, including myofiber atrophy and centralized nuclei (Fig. 7). The histological hallmarks of DM patient biopsies are sufficient to distinguish DM from other myopathies and even differentiate between DM1 and DM2. For example, disease specific biochemical fiber-type histopathology is predictive of DM1 versus DM2 (308). While genetic analysis is currently the only supported diagnostic measure, technical challenges in genetic testing for DM2 have resulted in a greater need for diagnostic biopsy. Thus, histological analysis of DM2 skeletal muscle is more common than in DM1.

In DM2, there is an increase in type II fibers and fiber atrophy appears greater in these compared to type I fibers (270, 423). In DM1, there is preferential type I fiber atrophy (423). Given electrical activity can stimulate fiber-type transitions, it is possible that myotonic discharges in DM influence fiber types. Fiber atrophy is preferential for distal muscle in DM1 and proximal muscle in DM2. Furthermore, within DM1 biopsies, type I fibers show preferential atrophy while type II fibers are more affected in DM2 (270). In DM2, pyknotic nuclear clumps—a marker of late atrophic myofibers—are observed.

The subsarcolemma positioning of myonuclei is a hallmark of mature muscle. Typically, myonuclei are regularly spaced within a myofiber and deviation from this normal organization patterns impairs muscle function (48). In several myopathic disorders and age-related sarcopenia, nuclei are mislocalized in the center of a myofiber upon histological examination. This is considered a marker of active regeneration and is associated with several congenital myopathies (182,183). Beyond a marker for myopathy, central nuclei may directly contribute to muscle dysfunction by disrupting sarcomere organization and affect myofibril contraction (113). Several regulators of myonuclear positioning are emerging and their contribution to centralized nuclei in DM has not yet been characterized (114, 260). While central nuclei are observed in both DM1 and DM2, DM2 patients tend to show a preference for central nucleation in type II fibers (308). However, as with other measures of DM histopathology, the frequency of central nuclei appears greater in DM1 (356,423). While less studied, other features of DM muscle histopathology include split fibers, ring fibers, late-stage fibrosis and steatosis, nuclear chains, and large/irregularly shaped myonuclei (133, 423).

CDM muscle

CDM patients display a variety of *in utero* and perinatal phenotypes, indicative of disrupted muscle development, and histopathological features throughout embryogenesis and postnatal life. Indeed, CDM muscle contains an increased proportion of immature myotubes, small muscle fascicles, central nuclei, myofiber size variability, and fiber-type disproportion (14, 108, 174, 193, 342, 343, 352, 369, 411).

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One of the first studies of CDM histopathology reported a reduction in IIB fibers and atrophy of type I fibers, similar to results obtained from adult DM1 biopsies (14). These authors suggested the involvement of dysfunctional motor neurons in these phenotypes, similar to congenital fiber-type disproportion disease (14). While abnormal motor endings have been observed in CDM, other groups have suggested normal innervation patterns in CDM (108, 343). Given the establishment and maintenance of mature neuromuscular junctions is dependent on both motor neuronand muscle-intrinsic mechanisms, both cell populations may play a role in disease (447). CDM infants are often born prematurely and muscles at 27, 34, and 37 weeks gestational age have been studied, revealing morphological and histochemical markers of fiber immaturity (342). Some patients displayed differences in satellite cell number and all three patients showed evidence of increased lysosome accumulation—a marker of fiber necrosis (342). Unfortunately, studies of CDM histopathology are often limited based on small sample numbers and lack of appropriate controls. Therefore, the current data regarding characteristics of CDM muscle should be interpreted with caution. This scarcity of data highlights the need for more thorough analysis of CDM patient skeletal muscle as well as the generation of animal models that can address the fundamental basis of myogenic defects in CDM. Although a variety of staining methods have revealed aspects of DM histopathology, additional work utilizing immunolabeling techniques should provide information regarding important myogenic cell populations, such as satellite cells, which have been proposed as being dysfunctional in CDM (122). Furthermore, cell-cell and cell-matrix interactions are essential for muscle development and may play a role in CDM manifestations (61).

Molecular Mechanisms Involved in DM Pathogenesis

In the previous sections, we provided an introduction into the DM field and highlighted key clinical and histological presentations of DM patients. Complex molecular mechanisms underlie these phenotypes and, in many cases, aspects of the disease are still under investigation. In the remainder of this review, we provide detailed information regarding the molecular pathogenesis of DM and how particular events relate to disease. In this section, we first discuss aspects of DM molecular pathology that are likely at play in any given cell type. We then focus our attention on studies exploring aspects of muscle development, function, and maintenance and the model organisms used to understand these processes. Given the prevailing view that disruption of RNA processing pathways is a central pathomechanism in DM, particular attention is given to these regulatory networks.

A fundamental question in understanding DM pathogenesis is how simple repetitive CTG and CCTG sequence motifs in noncoding regions of the genome give rise to the variety of features described above. The complexity of this question is underscored by several observations. First, DM is a multisystemic disease with nearly every organ system in the body affected to some degree. Second, given the location of the *DMPK* and *CNBP* C(C)TG^{exp} repeats in noncoding regions, it is unlikely DMPK or CNBP gain-of-function mediated through alterations in amino acid sequence or loss-of-function resulting from frame-shifting would contribute to disease. Third, the striking genetic anticipation of DM1 suggests a repeat toxicity dose effect. Finally, and perhaps most intriguing, the partial phenocopy between DM1 and DM2 hints at some overlapping disease mechanism.

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Two main pathogenic mechanisms have been proposed. First, C(C)TG^{exp} mutations may alter the expression patterns of *DMPK*, *CNBP*, or neighboring genes through epigenetic mechanisms. Second, DM is an RNA-mediated disease and C(C)UG^{exp} RNAs are toxic through the modulation of downstream effectors. As explained below, the latter RNA gain-of-function mechanism has emerged as the predominant contributor to molecular toxicity. However, cooperativity may also exist between these mechanisms, particularly in the case of CDM where highly expanded repeats are present throughout embryogenesis. In this section, we will survey the fundamental principles of genome-, transcript-, and effector-level mechanisms of DM molecular pathology as they pertain to any tissue. In the next section, we will address molecular dysfunction as it relates to specific defects in skeletal muscle.

Genome level

DM is one of over two dozen microsatellite expansion disorders, and lessons learned from these other diseases can inform mechanistic theories of DM pathogenesis. For example, FXS is caused by a CGG microsatellite expansion in the 5' UTR region of the *FMR1* gene and results in intellectual disability, psychiatric dysfunction, and other neurological impairments (21). CGG^{exp} modulates epigenetic modification of this locus, including altered methylation and histone modifications, ultimately silencing *FMR1* transcription and eliminating the production of FMRP protein—a key regulator of local translation in neurons (21). CTG^{exp} elements influence nucleosome positioning, suggesting transcriptional dysregulation of the *DMPK* locus may also be involved in DM pathogenesis (424). Indeed, increased nucleosome occupancy was preferentially observed on mutant *DMPK* alleles and appeared to correlate with repeat number, suggesting transcriptional disruption of the *DMPK* locus may be involved (433). The *DMPK* 3' UTR contains a DNase I hypersensitivity site in the wild-type allele, but shows resistance to DNase I cleavage in DM1-patient-derived cells and tissues, indicative of heterochromatin (287). Furthermore, CpG-islands are located upstream and downstream of the CTG^{exp} that are unmethylated in normal adults and most adult DM1 patients, but show frequent hypermethylation in CDM (380). This result indicates a unique contribution of highly expanded *DMPK* alleles to certain epigenetic modifications and also provides one of the first molecular distinctions between adult-onset DM1 and CDM. The spread of heterochromatin at this locus may be restricted by two CTCF-dependent insulator regions flanking the CTG^{exp} (74, 112). While *DMPK* hypermethylation is postulated to disrupt CTCF-binding and chromatin condensation near the CTG^{exp}, CTCF occupancy at this locus appears to be methylation-insensitive (74, 235, 451).

The *DMPK* CTG^{exp} mutation resides in a gene-rich region. The *dystrophia myotonica WD repeat-containing protein* (*DMWD*) and *sine oculis homeobox homolog 5* (*SIX5*) are upstream and downstream of *DMPK*, respectively, and reside within an ~30 kb window. Both of these genes are expressed in developing and mature muscle. The *DMPK* 3' UTR overlaps with putative *SIX5* promoter elements and the DNase I hypersensitivity site lost in DM1 serves as an enhancer element (200). *SIX5* expression is reduced in DM1 patient-derived fibroblasts, myoblasts, muscle, and heart tissue (200, 402). This reduction is allele-specific and correlates with repeat number (200, 402). Additionally, *DMPK* RNA and protein levels were originally reported as being decreased in DM1 patients (120). As DM is inherited in an autosomal dominant fashion, this would support a haploinsufficiency

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model (120). While this model is not expected to agree with the anticipation observed in DM, it is possible that repeat length-dependent increases in epigenetic changes would result in a step-wise decrease in RNA and protein production of DM1-linked genes. However, hypermethylation of the *DMPK* CTG^{exp} proximal locus does not correlate with repeat length (235). Rather, the hypermethylation status appears specific to CDM and suggests the presence of pathogenic allele sizes during development triggers the establishment of epigenetic changes that cannot be recapitulated during postnatal repeat expansion (235). Recent work using a large cohort of patient blood, chorionic villus, and human embryonic stem cell (ESC) samples confirms hypermethylation adjacent to the *DMPK* CTG^{exp} is a prominent, and unique, feature of CDM (24). Additionally, the authors report that methylation upstream of the CTG^{exp} is unique to maternally derived germ cells, which may explain the transmission bias of CDM alleles from mothers (24). In male spermatogonia, methylation-induced reduction in *SIX5* expression may result in loss of these cells, and this may explain the exceedingly rare paternal transmission pattern. In agreement, *Six5* KO mice display male infertility associated with impaired post-natal spermatogenesis (351). Because germ cells are haploid, CTG^{exp}-induced gene silencing would be expected to be more deleterious than in diploid cell populations.

The most compelling evidence against a DM haploinsufficiency model comes from a variety of heterozygous and homozygous mouse KO studies (Table 1). Heterozygous *Six5* KO mice are normal and do not support a model whereby partial loss of SIX5 recapitulates DM phenotypes (199). While homozygous *Six5* KO mice develop cataracts, they do not resemble the subcapsular particulate cataracts present in DM patients (199). Heterozygous *Six5* KOs may display subtle cardiac abnormalities, but a direct link to DM pathology is unclear (427). To date, reduced SIX5 protein levels have not been demonstrated in DM tissues. However, as mentioned above, reductions in SIX5 levels may be restricted to, and particularly toxic in, haploid cells such as male germ cells.

Although mechanistically unexplored, the CTG^{exp} may affect the expression of *DMPK* itself. Furthermore, the presence of 3’ UTR CUG^{exp} tracts could theoretically disrupt other aspects of DMPK RNA metabolism such as nuclear export and/or translational efficiency. To test the contribution of *DMPK* loss-of-function to DM1, heterozygous and homozygous *Dmpk* KO mice were generated (327). Heterozygous KO mice are overtly normal, with no reported overt abnormalities or decreased life expectancy (327). Later studies reported cardiac abnormalities but recent work suggests normal cardiac and muscle function in various DMPK-depleted mouse models (30, 62). Homozygous KO mice were originally described as having mild, late-onset myopathy but again, recent studies do not support these original observations (62, 179, 327). Along with a lack of robust DM-relevant phenotypes in mouse KO studies, the original evidence of reduced *DMPK* mRNA and protein levels is controversial and may be associated with technical artifacts including RNA purification techniques and quality of anti-DMPK antibodies (85, 124, 147, 215, 240, 432). As mentioned above, use of cesium-chloride gradient RNA purification strategies recovers normal *DMPK* mRNA levels in DM1 samples compared to controls (85). While *DMWD* loss-of-function studies have not been thoroughly conducted, *DMWD* mRNA levels are not reduced in patient-derived fibroblasts (147). Overall, these results fail to support a major contribution of *SIX5*, *DMPK*, or *DMWD* haploinsufficiency to DM1.

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Unlike DM1, the CCTG^{exp} in DM2 is located far from neighboring genes, suggesting no gene besides *CNBP* would be susceptible to haploinsufficiency in DM2. CNBP protein is localized to the nuclei of embryonic mouse tissues where it promotes cell proliferation partly through transcriptional activation of c-*MYC* (68, 365). Although controversial, studies have shown CCTG^{exp}-associated decreases in *CNBP* expression levels (316,345). Homozygous *Cnbp* KO mice are embryonic lethal, and present with dramatic developmental abnormalities including largely absent forebrain and craniofacial defects (Table 1) (68). Interestingly, *Cnbp* heterozygous KO mice recapitulate electrical myotonia, cardiac conduction defects, cataracts, and myopathic features (69). CNBP is typically expressed at high levels in skeletal muscle and is localized to Z-lines (316). In DM2, the *CNBP* expression pattern may be altered and may affect the translation of CNBP-target RNAs, several of which have known roles in muscle function (172,316). A key distinction between DM1- and DM2-associated microsatellite expansions is the location of the repeat. In DM1, the CTG^{exp} in the 3' UTR of *DMPK* is preserved after mRNA processing, yielding capped and polyadenylated transcripts. Other pathogenic microsatellites, such as the CTG^{exp} in Fuchs endothelial corneal dystrophy (FECD) (442), the GGGGCC^{exp} in C9-ALS/FTD or the CCTG^{exp} in DM2, reside in introns of *TCF4*, *C9orf72*, and *CNBP*, respectively, and therefore are expected to be completely spliced out of the final mRNA population. In this context, the spliced intron would be less stable and the mature mRNA would be spared from downstream disruption. The expected processing pattern (i.e., intron 1 splicing) of mutant *CNBP* transcripts is supported by early studies (249), in contrast to recent findings in C9-ALS/FTD, in which the GGGGCC^{exp} led to intron retention in patient lymphoblasts (275).

Knowledge regarding the normal functions of the DMPK, SIX5, and CNBP proteins is important, as this information may provide insights into the molecular defects associated with their possible loss-of-function in patient tissue and animal models. DMPK is a serine/threonine kinase and is localized to the nuclear envelope of HeLa and C2C12 cells (153, 185). Overexpression or knockdown is associated with altered laminin protein levels and localization in these cells and disruption of myotube formation in C2C12 cells (152, 153). DMPK may also support resistance to reactive oxygen species (ROS) and antagonize ROS-induced cell death (292). CNBP is a single-stranded RBP and DNA binding protein and has been suggested to bind to genes associated with Wnt signaling pathways (248). SIX5 is a homeodomain protein, a protein family essential for embryonic development, and its misregulation would be expected to exacerbate myopathy (46).

Transcript level

Transcription across C(C)TG^{exp} DNA generates C(C)UG^{exp}-containing RNAs. Early studies proposed that CUG^{exp} RNAs contribute to DM1 (63, 405, 432). According to this model, highly expanded C(C)UG^{exp} would disrupt cellular pathways leading to disease manifestations possibly via some RNA gain-of-function mechanism.

In the context of DM1, mutant *DMPK* transcripts are expressed and undergo normal pre-mRNA processing (i.e., 5' cap addition, splicing, and polyadenylation), but these mRNAs are selectively retained in the nucleus of DM1 muscle and fibroblast cell lines (85, 147).

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Retention is length-dependent, as CUG^{80} repeats are more often found in the cytoplasmic fraction than CUG^{400} repeat-containing transcripts (147). Beyond nuclear retention, CUG^{exp} RNAs are localized as punctate inclusions, or RNA foci, that also increase in DM1 myoblast cell lines containing longer repeats (391). As discussed more below, RNA foci are complex structures comprised of $C(C)UG^{exp}$ RNA and RBPs which can be compact and crowded structures depending on mutant RNA repeat length and copy number as well as the total amount of MBNL available to bind (386). Recently, CUG^{exp} RNA has been observed undergoing phase transitions to form viscous, gel-like structures *in vitro*, and these structures can merge, divide, or completely dissolve over time (178, 386).

A variety of transgenic mouse lines expressing CUG^{exp} transcripts support a role for toxic RNAs eliciting disease symptoms. One transgenic mouse model, generated using an ~45 kb human mutant transgene containing a CTG repeat expansion, underwent intergenerational expansion to yield a variety of large repeat mice (Table 1) (137). DM300 mice, a derivative of the original line, recapitulate features of DM1 and those with larger (1200–1800) repeats, show a more severe phenotype (132, 361, 362). However, the expression of these transgenes is low, likely below the normal levels observed in affected DM1 tissues, and therefore underestimates the contribution of CUG^{exp} RNA to disease progression. A direct role for CUG^{exp} RNAs in disease progression is most convincingly demonstrated in a mouse model expressing CUG^{250} transcripts under the control of a human skeletal actin promoter (*HSA*^{LR} mice) (245). These mice develop centralized nuclei, myotonia, and CUG^{exp} RNA foci that correlate with transgene expression level (245). Importantly, these observations reveal CUG^{exp} RNAs exert toxicity and recapitulate aspects of the disease independent of gene context. A variety of additional repeat mouse models have confirmed and extended these findings, many of which show muscle atrophy, myotonia, and heart defects (284).

Nuclear retention of mutant *DMPK* and *CNBP* transcripts is not the consequence of disrupted pre-mRNA processing (147, 249). Furthermore, the punctate localization of RNA foci suggests the involvement of coalescing factors recruited to these transcripts (261, 406). The first of these was identified as CELF1/CUGBP1 (406), but despite its interaction with CUG^{exp} RNAs *in vitro* CELF1 does not colocalize with RNA foci in patient tissues, suggesting that its sequestration does not occur *in vivo*. On the other hand, other CUG^{exp} binding proteins, most notably members of the MBNL family colocalize with RNA foci and directly interact with $C(C)UG^{exp}$ RNAs *in vivo* (105, 106, 134, 263, 386).

Effector level

Modulation of CELF and MBNL activities is the most significant and widely supported cause of DM pathogenesis (73, 221, 311). *In vitro*, CELF proteins preferentially interact with CUG^{exp} or UG-enriched RNAs (110,396,406). However, CELF1 does not colocalize with RNA foci *in vivo* (105, 106). Instead, CELF1 levels are increased in DM1 skeletal and cardiac muscle (188, 307, 407). CUG^{exp} RNAs can directly stimulate CELF1 increases through PKC-dependent hyperphosphorylation and stabilization of CELF1 protein (403) and transgenic *DMPK* CTG^{960} mice, which display cardiac abnormalities and increased CELF1 levels, show amelioration of symptoms and reduced CELF1 protein following PKC inhibition (211, 431). Other regulators of CELF1 steady-state levels include GSK3β, cyclin

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D3-CDK4, and calcineurin (180,324). CELF1 levels are also increased in regenerating and denervated muscle fibers (285, 392). Since features of regenerative myogenesis occur in DM1 muscle, is the increase in CELF1 levels simply a consequence? In support of a direct role, CELF1 levels are increased in the CTG^{960} mouse model prior to the onset of overt histopathology (284). Immunofluorescent labelling of CELF1 also revealed increased levels in mature, nonregenerative myonuclei of this mouse model, implicating CELF1 upregulation as a contributor to DM1 pathology (285). Additionally, CELF1 repression ameliorates myopathy in DM1 models (29).

MBNL proteins are orthologs of *Drosophila* muscleblind (Mbl) and in mammals, three MBNL paralogs exist, MBNL1, MBNL2, and MBNL3 (294). All three mammalian paralogs bind to CUG^{exp} RNAs *in vitro* and colocalize with RNA foci *in vivo* (105, 106, 263, 386). MBNL proteins associate with CUG^{exp} RNAs by binding GC steps interrupted by unpaired pyrimidines (DM1, U-U mismatches in CUG repeats; DM2, C-U/U-C in CCUG) and are stabilized via homotypic interactions mediated in their C-terminal domains (454). All MBNL paralogs bind CUG repeats *in vitro* with very high affinity and with even higher affinity to RNA fragments containing CCUG repeats (203, 386). This feature, together with higher expression of the *CNBP* gene, should evoke stronger MBNL-dependent spliceopathy in DM2. However, MBNL sequestration is likely to be limited by rapid turnover of spliced *CNBP* intron 1 (249). In contrast to CELF, *in vitro* MBNL binding is proportional to CUG repeat length (263). MBNL1 knockdown reduces the number RNA foci in DM1 patient cells (84) and MBNL overexpression increases the number and size of RNA foci *in vitro* (386). In adult muscle, MBNL1 is highly expressed and predominantly localized throughout the nucleoplasm. In the presence of $C(C)UG^{exp}$ RNAs, MBNL1 is redistributed to RNA foci where it is thought to be functionally inactivated. When CUG^{exp} size is small, or in cell populations where *DMPK* transcript copy number is low, excess MBNL leads to saturation of binding sites in foci and MBNL can be rapidly exchanged between foci and the nucleoplasm (165, 315, 386). In these instances, MBNL functional inactivation and target missplicing are relatively low. In instances where CUG^{exp} size increases (e.g., inter- or intergenerational repeat expansions), or in tissues with high *DMPK* expression, MBNL proteins are effectively titrated from the nucleoplasm and tend to circulate within foci between available binding sites (386). In agreement with this model, many DM-relevant splicing events show strong dose-response relationships between nuclear MBNL concentration and target exon inclusion levels (425). Furthermore, using a metric of inferred MBNL concentration based on >40 validated splicing events, the severity of spliceopathy in DM1 muscle can be accurately predicted (425). Together, these data are consistent with the model that increased sequestration of MBNL leads to progressive severity within DM patient populations and when critical thresholds are reached, may explain the distinguishing pathogenic features of presymptomatic, adult, juvenile, and congenital forms of DM1. In support of a direct role of MBNL in DM pathogenesis, AAV-mediated overexpression of MBNL1 in HSA^{LR} muscle ameliorates myotonia, restores CLCN1 protein levels, and corrects missplicing (190). In a separate approach, a transgenic MBNL1 overexpression mouse corrects DM-associated pathology when bred to HSA^{LR} mice (64). Furthermore, *Mbnl1* KO mice recapitulate several DM associated phenotypes including myotonia, subcapsular cataracts, and histopathology (189). MBNL1 protein levels

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increase in postnatal muscle whereas CELF1 levels typically decline >10-fold, expression patterns that reinforce the pathological nature of their misregulation in DM (188). As discussed in more detail below, this pattern agrees with functional antagonism between members of these protein families.

Beyond MBNL proteins, other constituents of RNA foci are described (306). However direct versus indirect binding events need to be carefully distinguished to determine the proximal contributors to disease rather than proteins associated with the RBPs that are directly bound to C(C)UG^{exp} RNAs. For example, hnRNP H normally functions in coordination with MBNL1 and CELF1 to regulate AS (296). Increased concentration of MBNL proteins in RNA foci could lead to an increase in colocalizing hnRNP H. Additionally, an important pathogenic hallmark of MBNL sequestration in DM is its depletion from the nucleoplasm in conjunction with colocalization with RNA foci. This suggests a high degree of functional inactivation for MBNL that may not be the case for abundant nuclear proteins such as hnRNPs. While colocalization with RNA foci is considered an important hallmark of RBP inactivation in DM, a variety of RBPs interact with CUG^{exp} RNAs *in vitro* and are misregulated in DM patient samples and mouse models. For example, Staufen1 (STAU1) is increased in DM1 patient skeletal muscle as well as *HSA*^{LR} and other DM1 mouse models, and increased STAU1 activity is associated with myopathic phenotypes in transgenic mice (83). While STAU1 interacts with CUG^{exp} RNAs *in vitro*, it does not colocalize with RNA foci *in vivo*, but may be associated with nuclear export of single CUG^{exp} RNA molecules (325). In agreement with the model that STAU1 interacts with nonfoci associated *DMPK* transcripts, increased STAU1 does not affect the number of foci in cell models or the association of MBNL1 with these structures (325). Interestingly, STAU1 also modulates the splicing of several DM-relevant transcripts including *INSR*, *CLCN1*, and many others, which may modify disease progression in DM (43). While STAU1 does not modulate RNA foci abundance, two other C(C)UG^{exp}-interacting RBPs, DDX5 and DDX6, reduce foci accumulation and rescue missplicing of some targets in DM cell models (181, 305). Furthermore, muscle histopathology is reduced in *HSA*^{LR} mice following DDX5 overexpression (181). As DDX5 and DDX6 are RNA helicases, it has been suggested that their activity unwinds structured CUG RNAs, making them more susceptible to turnover. As RNA foci are increasingly appreciated as complex structures, it is likely a variety of RBPs remodel these structures in a step-wise manner (178, 386).

Models and Modulators of DM Myopathy

In the previous sections, we discussed features of DM skeletal muscle functional and structural pathology followed by an overview of molecular mechanisms whereby C(C)TG^{exp} mutations elicit abnormal cellular responses. RNA toxicity mediated through the modulation of MBNL and CELF activities has emerged as the prominent contributor to disease pathogenesis. Although reduced expression of C(C)TG^{exp}-linked loci may partially contribute to disease, in the remainder of this review, we will primarily focus on RNA-toxicity-associated events with discussion of alternative hypotheses where appropriate.

The goal of dissecting molecular lesions downstream of C(C)UG^{exp} RNA toxicity is twofold: (1) comprehensively reveal the extent of cellular dysfunction in each affected cell

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type; (2) establish links between molecular dysfunction to patient symptoms with the hope of identifying avenues for therapeutic intervention. The latter objective has been achieved and is most thoroughly exemplified by CLCN1 splicing errors eliciting myotonia. However, the molecular basis of other symptoms, such as impaired myogenesis in CDM and adult muscle wasting in DM1, has proven more elusive. Answers to these unresolved questions may lie in identifying subtle changes in several components of complex pathways. To address this, the use of high-throughput and increasingly unbiased sequencing technologies is lending a comprehensive and detailed view of disease relevant pathways. Indeed, the scope and resolution of RNA-seq afforded by increased sequencing depth and read length is revealing the dramatic extent of RNA processing errors in DM patients and animal models. Many of these changes have likely been overlooked using less-sensitive and lower-throughput experimental strategies, underscoring the importance of developing and utilizing new technologies for interrogating patient transcriptomes. While generating data is one step, deconvoluting the drivers of myopathy from passenger events is a complex task. To this end, the use of sophisticated computational and statistical tools is allowing the dissection of complex pathways and providing resources to develop global views of dysfunction. Below, we focus on three aspects of muscle biology disrupted in DM: development, function, and maintenance. We discuss key models used to understand these pathways, RNA processing networks associated with each, and where possible, specific events linked to dysfunction.

Muscle development

The development of muscle, myogenesis, is a multistep differentiation process whereby muscle precursor cells (MPCs) differentiate into mature, contractile myofibers (Fig. 8) (28,455). Two major forms of myogenesis occur *in vivo*, developmental and regenerative myogenesis, with many common cellular intermediates and molecular requirements (7, 28). While there are differences between periods of myogenesis, a common cellular prerequisite for any stage is the myoblast. Myoblasts undergo extensive proliferation prior to an initial differentiation event into a myocyte, a migratory cell with enhanced cell adhesion properties (2, 3). Myoblasts fuse to generate new myofibers, or fuse with preexisting myotubes to increase muscle bulk. In *Drosophila*, specialized fusion competent myoblasts initiate this process, although a homologous cell type has not yet been identified in vertebrates (313). Prior to fusion, myoblasts exit the cell cycle and commit to myogenic differentiation. Hundreds of cell fusion events eventually give rise to multinucleated myofibers, which then undergo a variety of maturation processes. For example, biogenesis of sarcomeres provides the contractile properties of muscle and coincides with relocalization of myonuclei to the cell periphery (113, 204).

In the mouse, developmental myogenesis occurs in successive waves. Myofibers first appear during embryonic, or primary myogenesis, beginning on embryonic day 11 (E11) followed by fetal or secondary myogenesis between E15.5 and E17.5. During developmental myogenesis, MPCs originate from the dermomyotome, a compartment of mesodermal cells adjacent to the notochord and neural tube of postgastrulation embryos. The cells migrate into the developing limb bud and establish the initial framework of skeletal muscle. By birth, muscle patterning is already established and myofibers mature through a combination of hyperplastic, cell-fusion, and hypertrophic processes (155). Postnatally, myofiber growth is

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largely dictated by hypertrophic mechanisms highlighted by increased sarcomeric protein production (101, 347). However, resident muscle stem cells, or satellite cells, can be activated by various stimuli, including muscle injury, to initiate a postnatal myogenic program. These cells also contribute to the basal maintenance of noninjured muscle and their decline is linked to age-, or disease-, related loss in muscle mass (194).

The molecular control of myogenesis has been most thoroughly studied in the context of transcriptional programs mediated by myogenic regulatory factors (MRFs) whose expression oscillates throughout myogenesis to control a finely tuned gene expression profile (28, 49). The MRFs are a family of four basic helix loop helix transcription factors, MyoD, Myf5, myogenin, and MRF4. The essential, combinatorial, and lineage specific contribution to myogenesis played by MRFs has been demonstrated using a variety of murine KO models. Myf5 and MyoD play redundant roles in early stages of the myogenic program followed by myogenin and Mrf4-responsive transcriptional programs driving myofiber maturation. Upstream of these, another set of transcription factors, the paired-box homeodomain proteins Pax3 and Pax7 control myogenic specification of early precursor cells and contribute to the satellite cell quiescence-activation axis.

Beyond transcriptional control of gene expression, co-/posttranscriptional mechanisms regulate myogenesis. Indeed, the degree of AS in the regulation of gene expression was first appreciated in skeletal muscle (232). Splicing patterns change throughout muscle differentiation and regions surrounding alternative exons are enriched for several well-characterized RBPs including CELF, MBNL, and RBFOX families (36, 413, 414). Furthermore, targeted mRNA decay controls transcript levels and helps to maintain satellite cell quiescence (107, 156). RNAs, such as miRNAs and lncRNAs are also emerging as another layer of myogenic control (277).

In the context of DM, studies of developmental myogenic defects are most applicable to CDM as overt neonatal musculature defects go undetected in adult-onset forms of DM1 and DM2. Several CDM manifestations suggest myogenic defects, including reduced fetal movement *in utero*, polyhydraminos (increased amniotic fluid related to insufficient swallowing by the developing child) and talipes (foot deformities). Given bone and skeletal muscle development are intrinsically linked, it is possible myogenic defects contribute to talipes (197). As discussed above, muscle biopsy reveals immature myotubes containing centralized nuclei, disorganized fibers, and small muscle fascicles (108). Small myofiber size suggests a growth deficiency mediated, in part, by reduced myoblast fusion during embryogenesis. Importantly, DMPK mRNA and protein increases during induction of human myogenesis *in vitro*, and DMPK protein levels decrease soon after birth (Fig. 8) (123). Interestingly, we have found that several genes associated with NMJ development and innervation show similar expression patterns during *in vitro* myogenesis, suggesting a potential role for DMPK in the regulation of innervation.

Myoblasts isolated from fetal CDM skeletal muscle display several markers of impaired function including reduced doubling time, lifespan, and fusion potential (122). These results are supported by other studies that demonstrated p16-induced senescence of CDM-derived satellite cells (34, 398). *In vitro*-derived CDM myotubes express immature myosin heavy

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chain isoforms (122). Of note, *DMPK* expression increases in differentiating myoblasts, suggesting that enhanced CUG^{exp}-mediated toxicity exists during myogenesis (397). Based on ChIP-seq data, this *Dmpk* expression pattern appears to be regulated by core MRFs (e.g., MYOD and MYOG) during intermediate stages of C2C12 differentiation (397). RNA foci are detected in DM1, DM2, and CDM-derived myoblasts and the *DMPK* CTG^{exp} is unstable in CDM-derived myoblasts, with a tendency toward expansions (122, 300). Furthermore, MBNLs colocalize with nuclear CUG^{exp} RNA foci in patient-derived or model myogenic cell lines, suggesting the DM RNA gain-of-function model holds true in these cell populations (105, 106, 263). Myogenic defects are also observed in DM1, but not DM2, patient-derived myoblasts (298). This raises two important questions. First, given *DMPK* CTG^{exp} expansions predominantly occur in postmitotic tissues in adult-onset DM1, is developmental myogenesis normal, but regenerative myogenesis impaired, in DM1? However, this is a difficult question to address in patients, given the challenges associated with acquiring myoblasts from presymptomatic DM1 patients as this necessitates prior knowledge that patients will eventually manifest disease. Second, the lack of *in vitro* myogenic defects in DM2 myoblasts suggests one possible mechanism for lack of congenital disease in DM2. Of note, CELF1 is upregulated in DM1 but not DM2 myoblasts, and there is evidence that CCUG^{exp} RNAs modulate CELF1 levels in other systems (298). However, RNA foci are observed in DM2 myoblasts with colocalization of MBNL1, snRNPs and hnRNPs, but not RNAPII, SC35, CStF, or PML, suggesting the formation of these foci occurs post-/cotranscriptionally (300). PLCβ1, a promyogenic factor, is upregulated in both DM1 and DM2, perhaps to provide functional compensation to DM MPCs (103). Another RBP, hnRNP H, is also upregulated in DM1 myoblasts, colocalizes with RNA foci, and antagonizes adult pattern insulin receptor splicing (296). ZNF37A, a transcription factor that is downregulated in DM1 myoblasts, is associated with myogenic defects and its expression is responsive to CELF1 levels (127). Unfortunately, studies of patient-derived cells are confounded by technical limitations including disease severity at the time of isolation, culturing conditions, use of primary/immortalized cell lines, and number of *in vitro* population doublings. Indeed, there are conflicting reports of increased and decreased numbers of satellite cells in CDM muscle biopsies (342, 343). The use of animal and cell models provides a partial solution and allows more detailed mechanistic studies.

In the mouse, *Dmpk* expression is first observed in the developing somites along the anteroposterior axis of E10.5 and E11.5 embryos (191). Somites originate between E8 and E9.5 in the mouse embryo, and contain some of the first myogenic lineage-committed cell populations (170). While DMPK has not been localized to subsomitic compartments, such as the dermomyotome, its substantial overlap using *in situ* hybridization techniques is striking and suggestive of early deficiencies in the myogenic program (51, 170). This suggests that the toxic effects of CUG^{exp} RNA expression occur at the beginning of myogenic specification. In agreement with the observed DMPK localization, *DMPK* regulatory regions display skeletal muscle enriched enhancer activity. Transgenic mice expressing a GFP-*Dmpk* 3' UTR reporter construct under its native regulatory elements shows expression beginning in somites and in a variety of embryonic muscle groups (382, 383). *Cnbp* is expressed in embryonic and adult mouse tissues (68, 365). Cnbp RNA is detectable by northern blot as early as E7 and is expressed throughout embryogenesis

(365). *In situ* labeling reveals staining in a variety of pregastrulation tissues, and the signal becomes increasingly enriched in the midbrain and forelimbs by E11.5 (365).

Expression of the human *DMPK* CTG^{exp} 3' UTR with as few as 57 CTG repeats impairs C2C12 *in vitro* myogenesis (11). Surprisingly, *in vitro* myogenesis is also impaired by a human *DMPK* 3' UTR construct that contains only 5 CTG repeats (31). This activity was mapped to a region 5' to the *DMPK* 3' UTR CTG^{exp} and is associated with increased CELF1 activity (9,31,341). This result underscores the importance of CTG^{exp} gene context in the emergence of certain aspects of disease. Of course, the presence of a phenotype in CTG^{5} experiments is unexpected given this repeat size is within the normal range in DM patients, but may reflect toxicity resulting from overexpression of small repeats. Furthermore, additional CTG^{exp} proximal *cis*-elements in the *DMPK* may exert some form of toxicity when these RNAs accumulate in the nucleus. In agreement, cultured myoblasts from transgenic mice overexpressing an unexpanded *DMPK* 3' UTR display fusion defects and increased steady-state levels of CELF1 (274). Transgenic mice expressing CUG^{11} RNAs in the context of the *DMPK* 3' UTR also show signs of myopathy in adult muscle and myogenic defects in cultured myoblasts (382). This myopathy is exacerbated in CUG^{91} RNAs expressed in the same context, suggesting that while CUG^{exp} RNAs are inherently toxic, their context within the *DMPK* 3' UTR is necessary to recapitulate all aspects of toxicity (382). Interestingly, while myogenic defects are observed in this mouse beginning at primary myogenesis, somite architecture appears normal, providing clues that pathology begins largely at the stage of myoblast differentiation (382). Conclusive evidence for inherent toxicity of CUG^{exp} RNAs was shown using the *HSA* CTG^{exp} mouse model (245). While control, CTG^{5} mice are normal, mice with $CTG^{>200}$ (*HSA*^{LR}) display adult-onset phenotypes including myotonia, RNA foci, MBNL sequestration, and RNA missplicing. Interestingly, these animals do not present with overt early developmental phenotypes despite high transgene expression. CELF levels are not upregulated in *HSA*^{LR} mice, which may be due to a lack of *DMPK cis*-elements required for CELF increase. Expression of *DMPK* under the correct regulatory regions is also likely to play an essential role in disease manifestations. Indeed, primary myoblasts isolated from *HSA*^{LR} muscle, where the CTG^{exp} is driven by the α-actin promoter, fail to accumulate RNA foci although these foci are induced following myogenic differentiation (our unpublished data). Mouse models constitutively overexpressing *DMPK* 3' UTR CUG^{exp} RNAs in a variety of tissues display embryonic lethality, while skeletal muscle conditional models display severe muscular atrophy and myotonia (284). Conditional expression of this same construct in heart results in early mortality due to arrhythmia and cardiomyopathy, recapitulating the other major cause of DM patient mortality (430). Both of these models display increased CELF1 levels, MBNL sequestration in RNA foci, and misregulation of many AS events (284). Interestingly, several of these splicing changes are not observed in *HSA*^{LR} mice, reinforcing the importance of CUG^{exp} RNA context and expression pattern. The inherent toxicity of CUG^{exp} RNAs has also been shown in zebrafish utilizing direct injection of CUG^{91} RNAs into the nervous system and skeletal muscle of embryos (410).

In mice, expression of *Mbnl1* overlaps with *Dmpk* in E10.5 and E11.5 somites and developing limb buds (191). Temporal expression patterns reveal an intriguing correlation between *Mbnl* expression and events associated with myogenesis. The expression of *Mbnl1*,

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Mbnl2, and *Mbnl3* peaks between E13.5–15.5, E17.5–18.5, and E11.5–15.5, respectively, in the developing mouse (191). Interestingly, the observed *Mbnl3* peak overlaps with a hyperplastic phase (E11-E15) of muscle development while *Mbnl1* peak expression occurs during a time of terminal myofiber differentiation (E12-E17). While *Mbnl1* and *Mbnl2* are both expressed during embryogenesis and in mature tissues, MBNL3 and CELF1 levels decline as cells differentiate (Fig. 8) (188). This *in vivo* observation is recapitulated in C2C12 myoblasts since MBNL1 and MBNL2 protein levels remain consistent throughout C2C12 differentiation while MBNL3 is lost soon after the induction of differentiation (Fig. 8) (312). MBNL3 is undetectable in adult skeletal muscle, but strikingly reemerges following muscle injury with particularly high levels during active myoblast proliferation (312).

In *Drosophila*, depletion of muscleblind (Mbl) proteins results in dysfunctional sarcomerogenesis due to impaired maturation of Z-lines (16). MBNL proteins regulate other developmental processes. For example, MBNL1 and MBNL2 regulate early differentiation events in embryonic stem cells (ESCs) and increased expression of MBNL1 and MBNL2 promote mature splicing events that activate ES cell commitment (149, 373, 419). In the heart, loss of MBNL1 disrupts normal valve development (81). On the other hand, CELF activity is typically most prominent in immature cells, reinforcing the antagonism observed between MBNL and CELF proteins.

One of the most thoroughly studied DM-relevant modulator of myogenesis is MBNL3. While MBNL3 is not detectable in mature skeletal muscle, or PAX7^{+}-satellite cells associated with single isolated myofibers, two isoforms—37 and 28 kDa—of this protein are observed in C2C12 and primary mouse myoblasts (312, 397). Steady-state levels of these proteins are rapidly reduced upon induction of C2C12 myoblast differentiation (312). On the other hand, MBNL1 and MBNL2 protein levels remain relatively constant throughout C2C12 differentiation (226, 252, 312). This suggests a specific role of MBNL3 in the control of early myogenic events—a hypothesis supported by an enrichment of MBNL3 target RNAs in pathways associated with cell cycle and myoblast fusion (3, 312). MBNL3 was originally identified as a negative regulator of myogenesis using a variety of knockdown and overexpression models (223–225, 225, 375). One study identified disrupted MYOD-dependent transcriptional networks and decreased total MYOD protein levels following MBNL3 overexpression in C2C12 cells, indicative of impaired myogenesis (225). Particularly, several differentiation associated gene expression markers are downregulated following MBNL3 overexpression in C2C12 cells (224). Currently, there is no evidence supporting direct MBNL3 binding to MYOD transcripts, including a lack of MBNL3 CLIP clusters (312). MBNL3 supports inclusion of the *Mef2* β-exon, coding for a region of the Mef2 transactivation domain, through binding of a region in intron 7 (224). Loss of this inclusion is correlated with delayed myogenesis in C2C12 myoblasts and can be rescued by overexpression of Mef2 β-exon containing constructs (224). Mef2 cooperates with MyoD to regulate early myogenic events and the β-exon inclusion isoform of Mef2 proteins results in an increased activation of target genes (224, 462). This missplicing event was also observed in DM patient skeletal muscle and cardiac tissue (224). Interestingly, Mef2 transcriptional networks are also disrupted in DM patient cardiac tissue and this has been explained by alterations in Mef2-responsive miRNAs (187). In contrast to data supporting MBNL3 as

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an antagonist of myogenesis, later studies suggested that siRNA-mediated knockdown of MBNL3 in C2C12 myoblasts delays *in vitro* myogenesis (312). While seemingly in conflict, other myogenic regulators are known to carefully balance myoblast differentiation dynamics in a manner similar to MBNL3. For example, constitutive activation or KO of beta-catenin disrupts myogenesis and is suggested to maintain a balance between cell proliferation and differentiation (336). Our group generated an *Mbnl3* isoform KO (*Mbnl3* $^{E2/Y}$) mouse, which displays age-dependent regeneration defects but not overt developmental defects (312). Other studies utilizing *Mbnl3* $^{E2/Y}$ KOs found similar age-dependent defects, but no developmental abnormalities (75, 76). Furthermore, the transcriptome of *Mbnl3* $^{E2/Y}$ muscle, as surveyed by RNA-seq analysis of E15 forelimb, is largely normal with no overt splicing changes and only subtle gene expression changes (312). Since MBNL3 binds predominantly to 3' UTRs, it is possible that this protein regulates APA or translation, mechanisms not explored in our original study (26, 76). Given the other prominent 28 kD MBNL3 isoform is upregulated and redistributed to the nucleus in *Mbnl3* $^{E2/Y}$ mice, these events may act as a compensatory mechanism that masks potential phenotypes (312). Recently, our group has generated an *Mbnl3* KO model (3KO) lacking both MBNL3 37 kDa and 28 kDa protein isoforms, and we have identified myogenic abnormalities in primary myoblasts isolated from these animals (397). These defects include failure to form mature, multinucleated myotubes *in vitro* following induction of differentiation as well as morphological abnormalities in cell spreading (397). Interestingly, recent work has described MBNL3 as a regulator of tumorigenesis partly through regulation of *Paxillin* (*PXN*) antisense transcripts. PXN protein is a component of focal adhesion complexes of nonstriated cells and dysregulation of its activity, or other cell adhesion components, may be linked to adherence defects in 3KO myoblasts. Another surprising feature of 3KO myoblasts is the emergence of adult pattern exon utilization in many DM-relevant transcripts including *Bin1*, *Mef2d*, and *Neb* (397). Because loss of MBNL activity typically results in reversion to developmentally immature splicing patterns, these observations were unexpected, and while they remain to be mechanistically explained, the relative splicing strength of MBNL paralogs may be linked (386).

The activity of MBNL1 and MBNL2 during mouse muscle development has not been extensively studied, but combined loss of these proteins results in embryonic lethality near E16.5, a period associated with terminal stages of primary myogenesis (227). While High-throughput sequencing-crosslinking immunoprecipitation (HITS-CLIP) data suggest that MBNL1 is predominantly an intronic binding protein in C2C12 myoblasts, it is primarily a 3' UTR binding protein in adult mouse skeletal muscle (428). This differential binding may be mediated, in part, by highly dynamic MBNL localization patterns during myogenic differentiation (166, 252). MBNL binding to target RNAs in C2C12 myoblasts regulates global AS regulation and mRNA localization that is conserved between mouse and fruit fly (428). *Mbnl1* KO mice do not display overt myogenic defects but this is likely due to compensatory upregulation and functional compensation provided by MBNL2 (227, 428). While MBNL2 loss alone has no obvious effects on muscle performance in mouse models, morpholino-mediated loss of MBNL2 in zebrafish results in paralysis and musculature defects (239). Further work should more closely analyze aspects of neonatal myopathy in *Mbnl* KO lines, particularly in *Mbnl1* and *Mbnl2* compound KO models.

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CELF upregulation has also been implicated in developmental abnormalities (163, 409). Two transgenic CELF1 overexpression lines were generated and showed dramatic developmental delays correlating with the expression level of the transgene (409). Mice with the highest transgene expression (approximately eightfold) died at birth and showed muscle histopathology (409). Interestingly, p21 is upregulated in these mice, which suggests premature cell-cycle arrest in myoblasts (409). To evaluate the tissue specificity of CELF1 overexpression, cardiac and skeletal muscle conditional transgenic CELF1 lines were generated producing approximately fourfold to sixfold higher CELF1 levels than endogenous (163). As with other CELF1 overexpression studies, developmental abnormalities were observed in this mouse including failure to thrive, histopathology and splicing abnormalities (163).

The cellular composition of skeletal muscle is complex and includes many nonmyogenic cell populations: lower motor neurons, junctional Schwann cells, fibroblasts, monocytes, pericytes, and capillary-associated cells (28). Sustained capacity for adult regenerative myogenesis is dependent on many of these, as satellite cells reside in a highly specialized niche important for quiescence and activation (267). Furthermore, nonmyogenic cell populations such as pericytes, have been shown to possess myogenic potential (91). During developmental myogenesis, cross-talk between motor neurons and myofibers regulates normal formation of both, and disruption is associated with impaired development and disease (87, 192). While these nonmyogenic cell populations are understudied in the context of DM, recent studies have suggested their importance, as MBNL1 regulates myofibroblast development and fibrotic responses (86). One possible avenue to circumvent the challenges of these rare cell populations in patients is using induced pluripotent stem cells (iPSCs) generated from DM1 patient fibroblasts that have already been utilized in other studies (448, 449). In animal models, synaptogenesis is impaired in a CTG^{exp} mouse with cooccurring neuronal RNA foci, behavioral abnormalities, and disrupted vesicular trafficking proteins (161). Although early studies suggested normal lower motor neuron morphology in CDM infants, CDM infants are hypotonic, a symptom typically associated with neurologic involvement (38). Using human ESC derived neuronal cultures, two *SLITRK* paralogs were found to be misspliced in DM1 and associated with defective neurite outgrowth and establishment of neuromuscular junctions in muscle coculture experiments (250). *Mbnl* homologs in *C. elegans* have also been shown to regulate synapse formation, highlighting conserved roles across species (374). These data invite the hypothesis that similar abnormalities exist in motor neurons throughout developmental myogenesis.

Developmental myogenesis is clearly affected in CDM, and likely during regenerative myogenesis in adult-onset DM (10). Many DM splicing biomarkers have been described, and it will be important to address if similar or unique changes are observed in CDM (272, 425). While many normal splicing transitions begin postnatally, there is evidence for groups of splicing transitions beginning *in utero* (188). In agreement, missplicing of *INSR* has been reported in several CDM quadriceps samples obtained from aborted or still-born fetuses (123). *BIN1* exon 11 missplicing has been thoroughly described as contributing to myopathy and CDM-patient-derived cells display missplicing of this exon (121). Importantly, *BIN1* mutations are associated with centronuclear myopathy, a congenital muscle disease (104). Furthermore, a muscle-specific isoform of *myotubularin-related 1* (*MTMR1*) increases

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upon induction of myogenesis and is misspliced in CDM and DM1 (53). A homolog of *MTMR1*, *MTM1*, is mutated in X-linked myotubular myopathy, a congenital myopathy with striking similarities to CDM including generalized muscle weakness, hypotonia, and perinatal lethality (218). *MTMR1* is more frequently misspliced in DM1 than DM2, and correlates with age-of-onset in DM1 patients (349). Importantly, misregulation of this exon is observed during *in vitro* myogenesis of patient-derived cells, confirming aberrant processing during myogenesis (349). Understanding the extent of RNA misprocessing in CDM tissues will provide valuable insights into disease pathomechanisms, and generate valuable biomarkers. Moreover, studies of altered RNA processing during myogenesis are increasing our basic understanding of posttranscriptional regulation of development, and this will provide useful information for other common and rare developmental myopathies (273). To address these last two points, transcriptome-wide analysis of CDM muscle samples, and identified hundreds of misregulated AS and polyadenylation events present as early as 3 months of age (397). Interestingly, most CDM missplicing events are also disrupted in DM1 patient muscle, but are more severely misregulated in the former (397, 425). Furthermore, many CDM-relevant exons appear to undergo developmental RNA isoform transitions that are completed by birth, suggesting that disruption of these events *in utero* would impair normal myogenic differentiation necessary to sustain life (397). In agreement, mouse MBNL loss-of-function *in utero* results in congenital spliceopathy and gene expression abnormalities characteristic of CDM as well as frequent perinatal lethality, respiratory distress, muscle histopathology, and failure to thrive in compound KO models (397).

Muscle function

Diverse functional properties are provided by skeletal muscle including thermoregulation, posture, respiration, metabolic function such as glucose storage, and most fundamentally, movement (119). In addition to its role in mobility, muscle is increasingly understood as an endocrine organ producing muscle-intrinsic cytokines or myokines (440).

The basic functional unit of muscle contraction is the sarcomere, a repeating unit of myosin, actin, and associated regulatory proteins positioned along the length of a myofiber (173). Mutations in a variety of sarcomeric proteins contribute to both adult-onset and congenital myopathies (173,282,317). The sarcomere is linked to the muscle membrane through additional structural proteins and ultimately connected to an intricate structural network in the extracellular matrix (ECM), which also plays essential roles in muscle contraction, signaling, and other aspects of muscle function (129). Again, several mutations in components of these structures are causative of human disease, most notably *DMD* mutations and DMD disease (129, 258).

Contraction is governed by events linking neurotransmission to activation of myofibril cross-bridge cycling. Electrical stimuli transmitted through motor neurons culminate with the release of acetylcholine, an excitatory neurotransmitter, at the neuromuscular junction. These stimulatory molecules traverse synaptic junctions and bind to acetycholine receptors embedded within the postsynaptic sarcolemmal membrane. Transmission of the endplate potential leads to an initial influx of excitatory sodium ions leading to a robust, uniform contraction of muscle. The resulting endplate potential stimulates a variety of calcium

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channels allowing the influx of calcium from extracellular and intracellular storage compartments. The binding of calcium to regulatory units of the sarcomeric thin filaments allows for ATP-dependent contraction prior to repolarization of muscle fibers and rest (56, 136, 216). While this scheme is generally true for all striated muscle, distinct functional requirements by certain fiber types (e.g., type I, IIA, etc.) or developmental time periods require unique specializations. For example, many developmental and myofiber specific *titin* isoforms exist and are controlled through AS (142, 288). Modulation of muscle RBPs or alterations in specific exon usage patterns, disrupts the structure and function of muscle (130, 297). Some RBPs, even those within the same family, appear to have importance in the function and maintenance of mature muscle, but may be dispensable for myogenesis. For instance, RBFOX2 controls early splicing transitions essential for myoblast fusion although depletion of RBFOX1 does not disrupt *in vitro* myogenesis and global splicing networks are largely unperturbed (370). However, conditional deletion of RBFOX1 in adult muscle results in histopathology, calcium mishandling, weakness, and spliceopathy (297). Despite >70% deletion of RBFOX1 in satellite cells in this same model, regenerative myogenesis is not affected (297). While the remaining RBFOX1-positive satellite cells may compensate, these data support the dispensable role for RBFOX1 in early myogenic cells.

The most profound functional defects associated with DM skeletal muscle are myotonia and weakness. As described above, skeletal muscle contraction is activated by excitatory neurotransmitters originating from lower motor neurons and disruption in excitatory signaling or muscle denervation can disrupt muscle function (192). While early stage DM1 patients are spared substantial muscle weakness, they present with a marked reduction in reflexes and this is suggestive of lower motor neuron impairment (338). Several of these patients also showed signs of reduction in several parameters of motor neuron electrophysiology (338). While the structural integrity of DM1 motor neurons has not been explored in detail, there are reports of hyperproliferation of synapses in DM1 patient-derived spinal cord neurons and decreased axon myelination in phrenic nerves (289). Other studies have reported normal innervation in a cohort of DM1 patients in contrast to markedly disrupted innervation in ALS (95). With this in mind, the transcriptome of DM1 and DM2 skeletal muscle is highly disrupted and this misregulation greatly contributes to muscle-intrinsic pathogenesis (210). High-throughput expression profile techniques, including microarray and RNA-seq, suggest hundreds of misregulated splice patterns are present in DM1 skeletal muscle, with over 20 events correlating with markers of disease severity, including muscle weakness (272, 425). Given DM1 and DM2 patients show differences in affected distal versus proximal muscle groups, it will be important to understand the degree of these splicing changes in different muscles. Furthermore, type I and type II fibers are differentially susceptible in DM1 and DM2, respectively. The use of laser capture microdissection technology has allowed the study of splicing changes and their effects in specific fiber types of DM1 and DM2 patients (348). While this study found no significant differences in splicing of *INSR* between fiber types, other RNA processing changes may be fiber specific (348). Of note, many splicing changes observed in DM1 are observed in other myopathies, and direct links between specific splicing changes and patient symptoms must be experimentally validated.

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Several transgenic and other forms of DM mouse models have been generated that possess DM-relevant functional deficits (131, 367, 435). *HSA*^{LR} mice develop myotonia at approximately 4 weeks of age, due to *Clcn1* pre-mRNA missplicing (245). As with DM patients, myotonia is first present in the absence of overt muscle wasting and histopathology. While subtle markers of histopathology (e.g., some central nuclei) are present at later stages in this mouse model, the lack of overt muscle wasting is surprising, particularly given the transgene has been measured as being >1000-fold higher than endogenous *Dmpk* (138). In fact, this study compared transgene expression levels in several mouse models, including DM500, DMSXL and Tg26, and found the expression level of the *HSA*^{LR} transgene to be higher than all other models (138). These data suggest the spatiotemporal expression pattern of CUG^{exp} RNAs and their sequence context are important for these phenotypes. The *HSA* transgene is not expressed in NMJ-associated nuclei, whereas human *DMPK* is expressed and RNA foci are observed in these subjunctional nuclei (Fig. 9) (437). In addition, RNA foci and MBNL sequestration are observed in lower motor neurons of human spinal cord (437). Given the importance of NMJ maintenance in muscle function, these expression differences may be critical in understanding DM pathology (37). Other RNA misprocessing events are also present in the *HSA*^{LR} mouse, such as APA abnormalities (26). However, the lack of overt muscle weakness in the *HSA*^{LR} model suggests that the APA changes present in this mouse are not sufficient to generate these phenotypes.

Transgenic CTG^{exp} mice containing additional human *DMPK* elements, overcome the limitations of restricted spatiotemporal expression. DM300 mice recapitulate myotonia and progressive muscle weakness (361, 421). At 5 months of age, no difference is observed between DM300 and wild-type littermates, but hindlimb muscle strength is reduced ~30% at 10 months, suggesting the accumulation of dysfunction over time (421). This weakness is recapitulated in various isolated muscle groups (421). Components of the ubiquitin-proteasome pathway are increased in these same mice, but a cause/effect relationship has not been established (421). Metabolic disruptions are also present in DM300 mice and correlate with the severity of *INSR* exon 11 missplicing (140). As skeletal muscle is the body’s largest reservoir for glucose, defects in glucose storage may be associated with the insulin resistance in DM (268). DMSXL mice, a derivative of the DM300 line, display growth abnormalities as early as 4 weeks when bred to homozygosity, but do not display this phenotype in hemizygotes likely due to low transgene expression levels (132). Only two DM-associated splicing targets were tested in this study and only one, Insr, showed missplicing (132). Large CTG^{exp} mice from this line, including DM600 and DMSXL, that survive into adulthood display respiratory dysfunction, diaphragm histopathology and RNA foci in the diaphragm and phrenic nerve (290, 291). These results are important given diaphragm weakness and respiratory distress are major causes of morbidity and mortality in DM1 and CDM. Another derivative of these lines, with approximately 550 repeats displays skeletal muscle weakness and atrophy along with activation of the proteolytic Fbox32-ubiquitin pathway (421). EpA960 mice allow for conditional cardiac or skeletal muscle expression of 960 interrupted CUG^{exp} RNAs (430). Upon transgene induction, mice develop cardiac or skeletal muscle abnormalities secondary to *Tnnt2* missplicing and CELF1 upregulation occur as early as 12 to 24 and 6 hours postinduction, respectively (430).

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Importantly, these time-course data link these molecular changes as occurring prior to the onset of overt symptoms.

MBNL1 proteins are major contributors to adult-pattern *CLCN1* splicing in human, and thus, *Mbnl1* KO mice display *Clcn1* missplicing, loss of membrane-associated CLCN1 protein, and myotonia (189). *Mbnl2* KO mice do not recapitulate these phenotypes, and this suggests a particularly important role for MBNL1 in the mature skeletal muscle (66). While its loss is not sufficient to generate overt myopathy in adult mice, MBNL2 is upregulated in *Mbnl1* KO muscle, its relative nuclear abundance increases, and it provides functional compensation (227). While constitutive *Mbnl1*; *Mbnl2* compound KO (DKO) mice are embryonic lethal, skeletal muscle conditional *Mbnl1*; *Mbnl2* DKO mice develop exacerbated myotonia and missplicing (227). In addition, these mice develop dramatic muscle wasting (discussed below) (227).

While linking specific RNA processing events to a given clinical symptom is difficult, a well-established link between *CLCN1* splicing and myotonia exemplifies the greatest success toward this goal (67, 246). On the other hand, muscle weakness can be due to a variety of events, making a direct one gene-one symptom link more challenging. While defective EC coupling and/or inherent defects in the contractile apparatus are expected to contribute to the DM muscle weakness, the major, and life-threatening, contributor to muscle weakness is the age-associated loss in muscle mass, or muscle wasting, observed in late-stage DM patients.

Muscle maintenance

Skeletal muscle wasting, or atrophy, is the loss of muscle mass and occurs in response to aging, disuse, and disease. The maintenance of muscle is dependent on the longevity of healthy myofibers and a muscle's inherent potential for self-repair. Typically, muscle atrophy is not associated with overt loss in the number of myofibers, but rather the degradation of proteins, particularly sarcomere-associated components, within myofibers. Myofibrillar components comprise 70% to 85% of muscle protein and are the primary target of catabolic ubiquitin-proteasome pathways (80, 89). Catabolism-dependent muscle wasting is often seen in age-related atrophy (sarcopenia) or cancer-induced atrophy (cachexia) (6, 42). Satellite cells also contribute to steady-state myofiber maintenance and regenerative myogenesis following injury. Importantly, satellite cell numbers decline as a function of age and this reduction is associated with sarcopenia. In cases of overt myofiber loss, a critical threshold of calcium mishandling is typically the major contributor to necrotic pathway activation in disease such as DMD (54). The downstream effectors of this calcium mishandling are typically calcium-dependent calpains as well as activation of ER stress response pathways (44,54). In these cases, a fibrotic and inflammatory response typically cooccurs.

Skeletal muscle atrophy accounts for approximately 60% of DM patient mortality, followed by cardiac-related mortality (157, 344). Given the lack of inflammatory cell invasion and fibrotic deposits in DM1, muscle wasting is likely due to loss of muscle mass independent of myofiber loss. In DMD, disruption of the sarcolemma is the prominent contributor to the calcium-mediated necrosis discussed above. In contrast, sarcolemmal integrity in DM and

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a variety of mouse models appears normal (133). Furthermore, elevation of serum creatine kinase, a marker of membrane permeability, is typically absent or unremarkable in most DM patients (133, 158, 159). While some studies have reported apoptotic markers in DM muscle, the contribution of apoptosis to muscle wasting is controversial as the proportion of lost nuclei is small compared to the number of nuclei found in mature myofibers (236). Many aspects of DM muscle wasting are similar to sarcopenia and this suggests that DM is a progeroid-type disorder (8). Muscle wasting in DM may result from activation of catabolic pathways downstream of a primary pathology such as missplicing, or from the direct role of DM-associated factors in translation (404). Indeed, both MBNL and CELF proteins have been implicated in direct or indirect control of mRNA translational efficiency. As previously discussed, missplicing of genes associated with other myopathies is prevalent in DM skeletal muscle tissues. The challenges of muscle maintenance in DM are compounded by the increased burden of age-associated increases CTG copy number in these muscle fibers (12, 266, 400, 444). The late stage onset of muscle wasting is likely linked to these increases in CTG^{exp} size.

Satellite cells are one of the major contributors to muscle repair in the context of disease (363). During normal development, a subset of Pax3/7 specific myogenic cells avoid MRF expression into adulthood and give rise to a subset of cells with a high degree of regeneration potential. Satellite cells are situated between the basal lamina and sarcolemma and are quiescent until activated by mechanical disruption or signaling events. Under normal circumstances of activation from quiescence, these cells undergo asymmetric cell divisions to yield cells that differentiate into myoblasts and cells that maintain the stem cell pool (283). Unfortunately, *in situ* studies of satellite cells in DM are limited and in general, challenging to study in the context of any muscle disease. However, *ex vivo* analysis suggests several aspects of satellite cells are dysfunctional in DM1 (398). For example, most studies of patient-derived cells suggest they undergo premature senescence that is independent of telomere shortening, but rather, may be associated with premature p16 activation (34). This suggests alterations of specific intracellular pathways. CELF1 is known to bind to *p21* mRNA and provides one possible explanation (408). In contrast, telomere shortening might impair the regenerative capacity of DM2 MPCs (329). Beyond satellite cells, a variety of nonmyogenic cells also contribute to muscle homeostasis and regeneration, but their contribution to DM pathomechanisms is largely unexplored. One notable exception is the contribution of MBNL1 to myofibroblast differentiation, but these cells have not yet been studied in a clinical context (86).

In mice, overexpression of wild-type human *DMPK* RNA results in centralized nuclei and type I fiber atrophy (274). These results reinforce the concept that *DMPK* cis-elements enhance the inherent toxicity of CUG^{exp} RNAs (9,382). While *DMPK* levels are not increased in DM1 patients, one possibility is that nuclear retention of CUG^{exp}-containing RNAs increases the local concentration of *DMPK* transcripts in the nucleus, effectively mimicking *DMPK* increased steady-state levels. As the CTG^{exp} is in the *DMPK* 3'UTR and full-length DMPK transcripts are retained in DM muscle nuclei, retention of CUG^{exp} RNAs inevitably results in an increase in the remaining DMPK mRNA sequence. Notably, HSA^{LR} mice do not exhibit overt muscle wasting, but rather show evidence of myofiber hypertrophy, which may be related to increased calcineurin signaling in adult animals (324). It is possible

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that the lack of native CUG^{exp} RNA flanking sequence or the normal *DMPK* spatiotemporal expression pattern may contribute to this absent phenotype.

Muscle-specific, combined loss of MBNL1 and MBNL2 results in dramatic adult-onset muscle wasting, with nearly 100% of fibers containing one, or multiple, centralized nuclei (227). Both quadriceps and TA muscles contain a mixture of type I and type II fibers, and both display muscle wasting in compound *Mbnl1*; *Mbnl2* DKO mice, invoking a contribution of MBNL proteins to maintenance of myofibers implicated in both DM1 and DM2 (227). Many splicing events known to contribute to muscle weakness and wasting, such as *Bin1* exon 11 and *Cacna1s* exon 29, are severely misspliced in these animals and may represent underlying contributors to this pathology (227). However, as these markers were assessed in end-stage animals, earlier time points should be studied to determine if these events are simply secondary to muscle regeneration (Fig. 10) (19, 285).

Overall, a variety of model organisms recapitulate hallmark features of DM myopathy. Importantly, DM-relevant RNA processing networks are dysregulated in these organisms including AS, APA, and RNA localization. Certain biological processes are enriched in datasets of misregulated RNA processing in DM, including components of the ECM and channels important for ion homeostasis, many of which are implicated in other channelopathies (17,96). Next, we discuss RNA processing events of particular interest based on their known association with DM phenotypes, common misregulation in DM patients and several models or involvement in other myopathies.

Potential Links: Genes and Phenotypes

Profiling of the DM skeletal muscle transcriptome has revealed misprocessing of hundreds of transcripts (26, 121, 202, 272, 281, 302, 428). Some of these abnormal splicing events might contribute to dysfunctional contractile and metabolic properties of DM skeletal muscle as exemplified by *CLCN1* missplicing and links to myotonia. Importantly, spliceopathy is highly variable between patients. Affected individuals differ in the total number of genes affected, particular exons disrupted and the degree of missplicing (272, 425). Some of these differences may relate to disease severity and progression. Indeed, the degree of missplicing for *BIN1* exon 11, *NFIX* exon 7, *DTNA* exons 11/12a, and other splicing events correlates with ankle dorsiflexion weakness in a cohort of DM1 patients (272). Separating disease-initiating from secondary misprocessing events is a topic of active investigation. KO studies provide information regarding the critical role of DM-relevant genes in the context of muscle development, function, and maintenance, but these studies fall short of providing information regarding the contribution of structural or regulatory changes induced by missplicing. Deciphering the specific role of differential exon inclusion requires targeted disruption of splice site selection, which may be accomplished using ASOs (121,323). Many misprocessing events are reproducibly identified in studies of DM patients and animal models, suggesting these are important events to consider. Furthermore, pathway analysis of groups of genes often reveals common themes in EC coupling, sarcomeric proteins and regulation of ion homeostasis. In this section, we will discuss some of these events as they relate to various aspects of muscle biology. While not an exhaustive list, these represent common themes in DM pathogenesis and are probably important contributors to

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myopathy. Where possible, we will discuss our current understanding of how DM-associated misprocessing of these RNAs have been experimentally validated to contribute to myopathy.

Migration, adhesion, and fusion in muscle development and DM

MPCs, including satellite cells, myoblasts, and myocytes, are a migratory population of cells. During developmental myogenesis, MPCs migrate from the dermomyotome into the developing limb bud prior to extensive proliferation and fusion to generate myofibers. During regenerative myogenesis, quiescent satellite cells are activated and migrate to points of injury (357). Satellite cells also move rapidly along isolated myofibers grown in culture (368). MPC intrinsic and extrinsic components are critical for migration and MPCs connect to a variety of collagens, laminins, and fibronectin of the ECM and transmembrane proteoglycans of the myofiber. MPC attachment to ECM components is largely mediated through transmembrane alpha and beta integrins, which are linked to a dense array of intermembrane proteins linked to actin filaments (Fig. 11). Collectively, these structures are known as focal adhesions, and these structures provide many signaling functions in addition to their roles in motility (264). These attachments provide traction points and act as "molecular feet" during migration. Focal adhesions are typically found in the posterior region of a cells leading edge and anterior to this is a branching array of actin protrusions forming lamellipodia and filopodia. These structures effectively push the membrane forward and are stabilized or recycled by a variety of actin binding proteins. As cells move, ECM components are also cleared, often by secreted metalloproteases, at the leading edge of migrating MPCs to provide space for movement (278). Extracellular chemoattractant molecules, such as IL-4, HGF, and PDGF, promote directional cell migration (3). Repulsive signals have also been described, such as ephrins embedded in the membrane of healthy myofibers which may promote MPC migration away from points where repair is not necessary (376). When two MPCs come in contact, or a single MPC contacts a potential site for fusion with a preformed myofiber, cell-cell adherens junctions form. These structures are similar to focal adhesions, and even share many of the same components, but are distinct in many ways, including adherens junction specific proteins, the posttranslational modification status of shared components and the fact that MPC adherens junctions establish the initial contact points necessary for cell fusion. A variety of signaling and accessory molecules such as β-catenin, FERMT2 (also known as kindlin-2) and a variety of small G-proteins are localized to MPC adherens junctions and are thought to be involved in signal transduction events necessary to initiate fusion (3, 94, 418). Upon induction of fusion, actin is rearranged at the sites of fusion, forming a dense network parallel to the cell membrane (97). The formation of this structure is dependent on nonmuscle myosin IIa (MYH9), an actin binding motor protein (97).

In DM muscle, transcriptome studies have largely focused on patient biopsy/autopsy skeletal muscle or *in vitro* differentiated myotubes (121, 272, 425). Therefore, transcripts important for MPC migration and fusion have not been specifically studied in the context of DM. However, many of the gene products disrupted in adult DM myofibers are utilized by MPCs for controlling early myogenic events. Fortunately, this provides *a priori* knowledge as to which transcripts are likely disrupted during these early stages of myogenesis. For example, many of the large array of actin-associated protein-coding transcripts are also expressed

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in MPCs and control focal adhesions, filopodia extension and cell-cell adherens junctions. Furthermore, CLIP-seq studies have identified the binding profile of MBNL1, MBNL3, and CELF1 in C2C12 myoblasts, providing a catalogue of potential pathways misregulated in DM patient MPCs. Among these pathways are processes associated with membrane dynamics, cell adhesion, cell-cell contact, and actin reorganization (312, 428). While the functional consequences of MBNL binding to these transcripts have not yet been explored in detail, prior studies suggest roles in localization and/or transcript stability (252, 428). These observations are supported by cytoplasmic MBNL1 and MBNL3 localization, particularly during the reestablishment of cell-matrix contacts following trypsinization and replating of C2C12 myoblasts (166, 312, 428).

In the following sections, we discuss a selection of gene transcripts implicated in DM myopathy grouped by functional pathways important for MPCs, contraction/muscle structure, and modulators of gene expression.

Cell adhesion and ECM components

PDLIMs—PDZ and LIM domain (PDLIM) proteins are a large family of cytoskeleton-associated proteins important for stabilizing and/or redistributing F-actin through associations with alpha-actinin and other actin binding proteins (416). Additionally, PDLIMs modulate protein phosphorylation and function via kinase activity found in their LIM domains (13). In the context of cell-migration, PDLIMs are known to phosphorylate cofilin, another actin mediator important for directional cell migration. Cofilin is localized to the leading edge of migratory cells and, when activated, severs actin filaments at the F-actin pointed end. This facilitates rapid depolymerization of F-actin into G-actin which can then be reincorporated into the barbed end of growing actin filaments—a process termed treadmilling. Loss of one PDLIM family member, PDLIM7, disrupts cell motility and is sufficient to cause perinatal lethality in mice (207,394). In addition to the multiple PDLIM paralogs found in mammals, a variety of splice isoforms exists, some of which regulate the abundance of certain PDLIM paralogs. For example, a splice isoform of PDLIM4 accumulates during stress that generates an unstable, and rapidly degraded protein isoform and despite its short half-life, this PDLIM4 isoform impairs cell migration (143). In DM, several PDLIM family members are misprocessed including PDLIM3, PDLIM5, PDLIM6 (also known as LDB3), and PDLIM7 (272, 425). While the function of these various splice isoforms has not been totally explored, LDB3 has received some attention (see discussion below).

CAPZB—Capping actin protein of muscle Z-line beta (CAPZB) subunit is a subunit of an F-actin cap binding complex. As with PDLIM, CAPZB is associated with actin filaments at the Z-line of mature muscle, but also participates in the regulation of actin dynamics during cell migration where it blocks the incorporation of actin at the barbed end (92). Propolymerization factors, such as cofilin, compete with CAPZB for barbed end interaction, and changes in local pH are speculated to shift the balance of interacting proteins (92). Interestingly, AS may adjust the pH sensitivity of actin binding proteins (92). In a cohort of patients presenting with hypotonia, micrognathia, and other developmental abnormalities, a chromosome 1 and 13 translocation, t(1;13)(p36.13;q12.11), has been

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identified that disrupted *CAPZB* expression (269). In agreement with a role in development, *capzb* is expressed throughout embryogenesis in zebrafish and loss-of-function mutants display disrupted muscle structure (269). When stimulated by force, local CAPZB levels increase to support focal adhesion maturation (209), and CAPZB loss-of-function impairs cell migration and filopodia morphology (371). CAPZB exon 8 is abnormally excluded in DM1 and DM2 patients, and missplicing is slightly more severe in DM1 patients (60, 188). Transgenic CELF1 overexpression mice also show missplicing of CAPZB exon 8 in developing heart and this event is not disrupted in *Mbnl1* KO mice (188). While this may represent an MBNL-independent splice event, functional compensation provided by MBNL2 may mask the contribution of MBNL loss-of-function (227). However, alterations in CELF1 and MBNL activities in DM skeletal muscle likely contribute to CAPZB exon 8 missplicing and may affect its ability to regulate actin polymerization in migratory cells and stabilize actin in mature muscle.

CLASP—Cytoplasmic linker associated protein 1 (CLASP1) is a microtubule-associated protein (MAP) involved in stabilizing microtubule plus ends along with a variety of other MAPs (168). In migratory fibroblasts, CLASPs are localized to the distal portion of microtubules at the cells leading edge where they are believed to stabilize microtubules via their associated with CLIP proteins (5). CLASP exon 19 is misspliced in DM1 patients and displays a broad range of missplicing that correlates with disease severity (425). While this exon has not been tested in current DM mouse models, another CLASP exon (exon 25a in mice), is disrupted in *Mbnl1* KO mice (457). The function of this exon has not been explored in detail, but its loss is expected to affect microtubule dynamics. Interestingly, microtubules are critical regulators of focal adhesion disassembly in migratory cells (379). Furthermore, microtubules are also believed to provide a conduit for MMP-containing vesicle trafficking to cell-ECM junction, allowing ECM remodeling during migration (379, 389).

ITGA3—Integrin subunit alpha 3 (ITGA3) is an integral membrane protein enriched at focal adhesions—sites of tight cell-ECM connection. The integrin protein family forms heterodimeric integrin-alpha and integrin-beta complexes that provide an essential link between the intracellular actin cytoskeleton and the ECM. Beyond mechanical support, integrins provide signaling roles, often mediated through a variety of intermembrane, integrin-associated proteins, such as focal adhesion kinase (FAK). Deletion of *Itga3* disrupts myogenesis as early as the myoblast fusion stage *in vitro* (50). MBNL2/MBL directly interacts with the ITGA3 3’ UTR and controls its localization to focal adhesions (4). This localization likely depends on the known ACACCC zip-code localization motif originally described as an essential motif for ACTB RNA localization. Interestingly, MBNL and CELF proteins also bind to the Actb 3’ UTR in C2C12 myoblasts, suggesting a global role in trafficking RNAs important for association with focal adhesions (312, 428, 429). In agreement, there is striking colocalization of MBNL2 with activated FAK in A549 cells, suggesting MBNL2, and associated RNAs, are actively shuttled to these regions (4). ITGA3 is the first transmembrane protein-coding transcript shown to be localized to a specific subcellular region, and it has been suggested that this allows for local translation at these junctions (4). If this is the case, disruption of MBNL-mediated ITGA3 localization

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by C(C)UG^{exp} RNAs would be expected to inhibit mature focal adhesion formation and myoblast fusion.

ITGB1—Integrin subunit beta 1 (ITGB1) is another major structural unit of integrin-associated adhesion complexes. As with *Itga3*, loss of *Itgb1* expression disrupts myoblast differentiation as well as sarcomerogenesis (358). Additionally, ITGB1 cooperates with FGF2, to maintain the satellite cell niche and decline of this activity is observed in satellite cells (SCs) from aged mice (335). While the localization of Itgb1 has not been studied, this RNA is a target of all three MBNL proteins in mouse embryonic fibroblasts (MEFs) (26). MBNLs bind slightly upstream of the distal Itgb1 PAS and MBNL loss results in preferential proximal PAS usage, suggesting MBNL proteins promote expression of the full-length Itgb1 3' UTR (26). The truncated Itgb1 3' UTR isoform lacks motifs for the HuR RNA-stabilizing protein so the mRNA half-life may be reduced. In agreement, single-nucleotide mutagenesis of MBNL binding sites in the *Itgb1* 3' UTR is sufficient to increase proximal PAS usage and lead to reduced protein production in luciferase reporter constructs (26). As with ITGA3, disrupted MBNL activity in the context of DM1 may lead to reduced ITGB1 protein levels and impaired cell-cell and cell-ECM adhesion complexes.

FN1—Fibronectin 1 (FN1) is a secreted component of the ECM. FN1, along with other extracellular proteins, provides scaffolding essential for developmental myogenesis, muscle patterning, and maintenance of the adult satellite cell. Loss of FN1 contributes to satellite cell depletion and age-associated muscle wasting (237). *Fn1* mRNA is another 3' UTR target of MBNL1 and MBNL3 in C2C12 myoblasts (312, 428) and siRNA-mediated knockdown of Mbnl1 and Mbnl2 in C2C12 myoblasts results in relocalization of FN1 away from the cell membrane toward the insoluble fraction of cell lysates (428). Using secreted luciferase reporters linked to the Fn1 3' UTR, the expression of CUG^{exp} RNAs is sufficient to decrease protein secretion (428). Importantly, this activity was rescued following MBNL1 overexpression, which indicates that MBNL-mediated localization of RNAs is important for secretion (428). Beyond localization, Fn1 and many other ECM-protein encoding transcripts are misspliced in DM1 animal models (96). Altogether, these results suggest that the ECM is compromised in DM1.

Myofiber structural proteins

DMD—*Dystrophin* (*DMD*) is the largest human gene and is composed of 79 exons encoding the 427 kDa dystrophin protein (Fig. 12) (35). Dystrophin is a component of a large dystrophin-associated glycoprotein complex that links the cytoskeleton and ECM of muscle and provides a scaffold for force transmission during muscle contraction, as well as transduction of extracellular-mediated signals to the muscle cytoskeleton (35) and DMD frame-shift and truncation mutations cause DMD. The DMD pre-mRNA splicing pattern is regulated during development and involves exons 71 to 74 and 78 and the splicing pattern of all these exons is altered in DM1 muscles (271). However, only missplicing of exon 78 has pathological implications because mice lacking exons 71 to 74 are normal (82). Aberrant exclusion of DMD exon 78 in adults shifts the open reading frame and alters the DMD C-terminus with a hydrophilic and positively charged β-sheet domain exchanged by a negatively charged amphipathic α-helix. This missplicing event compromises muscle

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fiber organization during contraction (323). Conditional *Mbnl1*; *Mbnl2* KO mice display aberrant splicing of *DMD* exon 78 (227). In addition to its function in mature myofibers, an unexpected role of DMD in satellite cells has emerged in the control of cell polarity and satellite cell fate decisions (99). It is possible that DMD exon 78 missplicing might lead to disruption of the activation-quiescence axis in DM satellite cells.

LDB3—Lim domain binding 3 (LDB3) is a PDLIM family member that is highly expressed in skeletal muscle. In mature myofibers, LDB3 localizes to Z-lines and interacts with alpha-actinin, an actin stabilizing protein, through its PDZ domain (109). Several LDB3 alternative splice isoforms exist, some of which lack LIM domains containing kinase activity involved in signal transduction (109). Various *LDB3* loss-of-function mutations have been identified in humans presenting with dilated cardiomyopathy (15) or distal myopathy (70). In the mouse, deletion of the *Ldb3* gene results in perinatal lethality (460). Interestingly, sarcomerogenesis and Z-line formation are not overtly disrupted in this animal, suggesting LDB3 is important for the maintenance of muscle rather than development (460). Interestingly, some cases of cardiomyopathy are associated with *LDB3* mutations that disrupt its interaction with PKC (15). In the context of DM1, *LDB3* exon 11 is abnormally included in mature skeletal muscle and in cells transfected with CTG^{exp} constructs (450). The emergence of an abnormal protein isoform was confirmed via western blot in this study (450). Furthermore, the *LDB3* exon 11 containing protein isoform exhibits reduced binding affinity for PKC proteins (450). It is possible that this reduced affinity partly contributes to PKC hyperactivation in DM skeletal muscle, and provides a possible link for CELF1 increase. Furthermore, while the ability *LDB3* exon 11 containing isoforms to properly maintain Z-lines has not been studied, it is possible that missplicing of exon 11 also directly contributes to muscle weakness in DM.

ABLIM1—Actin binding LIM protein 1 (ABLIM1) is highly expressed in skeletal and cardiac muscle (333). In cardiac muscle, ABLIM1 localizes to Z-lines where it likely interacts with other actin binding proteins via its LIM domain to stabilize actin filaments (333). However, the exact function of this protein has not been explored in detail. ABLIM exon 11 is fully excluded in fetal skeletal muscle and inclusion gradually increases during postnatal muscle development (281). In a cohort DM1, exon 11 is completely excluded in the majority DM1 patients and in *HSA*^{LR} skeletal muscle (281). This results in an in-frame loss of 28 amino acids downstream of one of ABLIM1’s LIM domains and is speculated to disrupt protein-protein interactions (281). Given exon 11 inclusion is specific to cardiac and skeletal muscle, this is expected to disrupt muscle-specific processes (281). Using an exon 11 minigene, it was shown MBNL and CELF proteins directly regulate this splice event in an antagonistic manner—MBNL and CELF promote inclusion and exclusion, respectively (281). This suggests missplicing of this exon is directly responsive to MBNL and CELF dysregulation and may affect sarcomere function in DM. In agreement, overexpression of CUG^{exp} reduced exon 11 inclusion in C2C12 cells (281).

TNNT3—Troponin T type 3 (TNNT3) is a fast-twitch skeletal muscle troponin subunit. Binding of calcium to regulatory domains within the troponin C subunit of the troponin trimer transmits conformational changes to tropomyosin leading to its displacement from

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myosin binding sites. Troponin T is the specific subunit that transmits the conformational change. Several *TNNT3* exons are alternatively spliced, contributing to subtle protein differences throughout development. In DM, *TNNT3* shows premature inclusion of a fetal (F) exon, and fetal exon inclusion has been reported to be more common in DM2 than DM1 (422). An orthologous exon is misspliced in *Mbnl1* KO mice suggesting disruption of MBNL activity in DM contributes increased fetal exon inclusion (189). As with other structural proteins disrupted in DM, the contribution of this missplicing event to disease progression requires further investigation.

Myofiber ion homeostasis and EC coupling

BIN1—Bridging integrator 1 (BIN1) protein is expressed in many tissues and functions as a regulator of actin and membrane dynamics (176). As nascent myotubes mature into terminally differentiated, functional myofibers, specialized structures including the sarcoplasmic reticulum (SR), transverse tubules, and myofibrillar components develop. In the mouse, these structures are first observed beginning with the SR at E14, followed by the first observable transverse tubules at E15 (388). BIN1 is an essential mediator of transverse tubule invagination and development (219). *BIN1* exon 11, encoding the protein's phosphoinositide-binding domain, is abnormally excluded in CDM patient-derived cells and DM1 skeletal muscle (121, 272). ASO-mediated exclusion of BIN1 exon 11 results in defective transverse tubule biogenesis and is sufficient to cause muscle weakness in mice (121). Interestingly, alterations in BIN1 are associated with several forms of human disease including both congenital (276) and adult-onset (41) forms of centronuclear myopathy. In the brain, altered CpG methylation upstream of *BIN1* is associated with Alzheimer's disease progression (88) and increased BIN1 expression is a modifier of Tau pathology (65). This suggests that BIN1 misregulation may also contribute to central nervous system defects observed in DM patients.

CACNA1S—Calcium voltage-gated channel subunit alpha 1S (CACNA1S) a multipass, transmembrane calcium ion channel located in the transverse tubules of skeletal muscle. CACNA1S is found only in skeletal muscle, where it participates in EC coupling (23). In DM1 and DM2, skipping of CACNA1S exon 29 increases with more severe exclusion in DM1 (393). This exon is undergoes a developmental splice transition between E16 and P10 in the mouse (393) and this may provide important functional characteristics for postnatal muscle. *CACNA1S* exon 29 encodes 19 extracellular amino acids (23), and a direct contribution of their loss to DM1 myopathy is supported by several lines of evidence: (1) the absence of this splicing shift in another muscular dystrophy, Facioscapulohumeral muscular dystrophy (FSHD); (2) a correlation between this splicing defect and TA muscle weakness; and (3) the appearance of a myopathy in wild-type mice followed morpholino-mediated exon 29 exclusion (393). Moreover, *Mbnl1; Mbnl2* muscle-specific DKO mice show a significant shift in Cacna1s splicing, supporting the combinatorial role of multiple MBNL proteins in DM pathogenesis (227, 393). CELF1 overexpression also promotes the exon 29 exclusion isoform, reinforcing the antagonistic relationship observed between MBNL and CELF regulated events (393). Missplicing of CACNA1S represents a common theme is DM spliceopathy, where many affected genes have known roles in ion homeostasis and are implicated in other channelopathies (17). In agreement with a loss of proper calcium

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homeostasis in DM muscle, increased cytoplasmic calcium levels were identified in DM patients with a greater affect in DM1 than DM2 (350). These levels correlated with the degree of splicing changes observed in DM muscle (350). Importantly, these splicing defects may have consequences beyond contraction defects, and may play a role in myofiber death in DM (see below).

ATP2A1—ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 1 (ATP2A1) (also known as SERCA1) is a transmembrane calcium reuptake pump located in the SR (303). Following myofiber excitation, calcium is released from the SR via the RYR1 channel, and ATP2A1 regulates the reuptake of calcium and supports relaxation following contraction. In DM, ATP2A1 exon 22 is abnormally excluded, resulting in truncation of a C-terminal domain and a portion of the mRNA 3' UTR. ATP2A1 exon 22 is also misspliced in a variety of mouse models including *HSA*^{LR}, *Mbnl1* KO, and CELF1 overexpression models. Importantly, overexpression of MBNL1 in *HSA*^{LR} skeletal muscle rescues this splicing defect and validates MBNL as an important mediator of this splicing event (190). The exon 22 lacking protein, termed ATP2A1b, is highly expressed in DM1 muscle and may be particularly enriched in type I myofibers (459). Missplicing of ATP2A1 is also believed to contribute to altered calcium homeostasis in cultured DM myotubes (350). In agreement, the calcium reuptake activity of ATP2A1a (exon 22 containing isoform) is approximately double that of ATP2A1b when expressed in cultured myotubes (459). Beyond DM, mutations in *ATP2A1* cause Brody disease, another disorder characterized by delayed muscle relaxation (280). Interestingly, this delay in relaxation is not myotonia, as action potentials are not present during relaxation (139). Excess intracellular calcium levels can cause ER stress, mitochondrial dysfunction, and the activation of calcium-dependent proteases (44, 54). Underscoring the importance of calcium regulation is the contribution excess intracellular calcium plays in myofiber necrosis in DMD (54). *ATP2A1* is one of three SERCA pumps expressed in muscle. Another, *ATP2A2*, is also misspliced in DM1 skeletal muscle and displays altered functional properties (459).

Myofiber metabolism and protein homeostasis

INSR—Insulin receptor (INSR, also known as IR) is a transsarcolemmal receptor that responds to the ligand insulin. INSR is a tyrosine kinase receptor that regulates glucose metabolism (184) and upon ligand binding, autophosphorylation promotes intracellular signaling events that lead to activation of gene expression, protein synthesis, and glucose metabolism (79). Skeletal muscle is the body's largest storage compartment for glucose and IR is a critical mediator. Normally, INSR undergoes a developmentally regulated splicing event with alternative exon 11 encoding a portion of the C-terminal domain (360).

IR missplicing generates an isoform with less affinity for insulin, IR-A (– exon 11) rather than IR-B (+ exon 11), and it has been suggested that this leads to increased blood glucose levels in DM patients (353). This splicing event is controlled by MBNL and CELF proteins and is also misregulated in *HSA*^{LR} muscle (84). The IR-A isoform also shows a greater affinity for IGF-II (another IR ligand) compared to IGF-I with implications for hypertrophic muscle growth. Upon binding of IGFs to INSR, key components of the Akt/mTOR pathway are upregulated, notably mTOR and GSK3β, while the atrophy-promoting transcription

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factor FOXO is downregulated. Interestingly, GSK3β, a modulator of mTOR anabolic activity, is increased in *HSA*^{LR} skeletal muscle and DM1 muscle cultures (180). Alternating GSK3β levels may be one therapeutic strategy to correct muscle weakness in DM patients (436). As with other RNA processing events, differences in the extent of IR missplicing exist between DM1 and DM2 (348).

PKM—Pyruvate kinase is a glycolytic enzyme produced as two major isoforms, PKM1 and PKM2, that differ in their metabolic activities and expression profile. For example, PKM2 is typically expressed in cells with high metabolic demands such as proliferating cells and is also an important modulator of tumor progression (77). PKM isoform choice is dictated by an AS event regulating mutually exlcusive exons 9 and 10. Alternative splice choice results in PKM1 (+ exon 10) and PKM2 (+ exon 9) differing in a 22 amino acid C-terminal domain that dictates whether the protein is constitutively (PKM1) or allosterically active (PKM2). As skeletal muscle differentiates, a developmental switch occurs from PKM2 in MPCs to PKM1 in myofibers (126). In DM1 patients, PKM2 expression is increased and correlates with altered glucose metabolism and type I fiber atrophy (126). Using ASO morpholinos to specifically disrupt this splice choice in otherwise healthy muscle results in an increase in glycolytic metabolism (126). This exon is specifically regulated by CELF1 overexpression, but not MBNL1 knockdown (126). Importantly, PKM2 reexpression is highly enriched in type I fibers and may explain fiber-specific muscle atrophy in DM1 (126).

Myofiber gene expression and RNA processing

NFIX—Nuclear factor IX (NFIX) is a transcription factor expressed in a variety of tissues, and in skeletal muscle it regulates an embryonic to fetal myogenic gene expression program (259). While NFIX is nearly undetectable in embryonic muscle and myoblasts, its expression greatly increases during fetal myogenesis (259). In fetal muscle, NFIX cooperates with transcriptional repressors to prevent the expression of embryonic genes, such as *Nfat2c*, another transcription factor important to activating embryonic genes (259). Skeletal muscle ablation of *Nfix* is sufficient to cause defective myoblast fusion and sarcomereogenesis (259). Additionally, *Nfix* regulates regenerative myogenesis partly through the control of myostatin expression (334). *NFIX* exon 7 is misspliced in DM patients, *HSA*^{LR} mice, and *Mbnl1* KO mice (96, 227, 272). While the functional consequences of this splicing abnormality have not been explored, increased exon 7 inclusion in DM may disrupt the association of NFIX with other transcriptional coactivators and result in disruption of prenatal myogenic gene expression. This would be expected to contribute to disrupted developmental myogenesis in CDM and adult regeneration deficiencies in adult DM. Interestingly, NFIX increases the expression of some genes (e.g., *Bgn*) (259) that are targets of MBNL proteins (428), suggesting cooperativity between multiple layers of gene expression dysfunction in DM.

MEF2D—Myocyte enhancer factor 2D (MEF2D) is a transcriptional coactivator and regulates several aspects of muscle differentiation (310). The splicing of MEF2D is regulated by several RBPs, including MBNL and RBFOX proteins (224, 337, 370). In DM, two MEF2D missplicing events are present: (1) β exon; (2) a change in mutually exclusive α1 and α2 exon usage. The Mef2d β-exon is excluded following overexpression of

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CUG^{exp} RNAs in C2C12 cells, suggesting this splicing event is susceptible to misregulation in DM (224). Furthermore, MEF2D β-exon inclusion is significantly reduced in DM1 skeletal muscle relative to controls (224). MEF2D cooperates with MYOD to regulate early myogenic events and the β-exon inclusion isoform of MEF2D protein results in an increased activation of target genes (222, 224, 310, 462). Strikingly, overexpression of the myofiber-specific MEF2D isoform (α1 and β exon positive) is sufficient to rescue myogenic defects in fusion-deficient RBFOX2 depleted cells (370). These data support a critical role for correct MEF2D splicing in muscle development. Other MEF proteins are disrupted in DM1, such as MEF2A, which shows reduced mRNA and protein levels in DM1 heart (187). Alterations in MEF2-regulated mRNA and miRNA pathways are also observed in DM1 heart (187), a finding consistent with another study demonstrating altered miR-1 processing and heart defects in DM1 (322). Interestingly, MEF2 proteins are activated in a mouse model of Becker syndrome, a nondystrophic congenital myotonia disorder (446). The activity of MEF2 proteins increases in response to increased intracellular calcium, and promotes fiber-type specific gene expression programs (445). It will be interesting to test if altered MEF2 activity in DM modulates fiber-type specific pathologies. Importantly, missplicing of MEF2 proteins is observed in other neuromuscular disorders, and has been suggested to be a compensatory, rather than pathologic, mechanism (20).

MBNL—While MBNL proteins have been extensively discussed above, their RNA processing is also misregulated in DM patients and would be expected to influence their roles in pre-mRNA AS and polyadenylation (26, 189, 428), mRNA stability (252, 429), RNA localization (428) and miRNA-1 biogenesis (Fig. 13) (111, 322). The three MBNL paralogs possess inherent functional differences, which are further modulated by presence of variable amino acid sequences encoded by alternative exons (386). MBNL1 exon 5, also referred to as exon 7, is alternatively spliced and modulates subcellular localization (412). In DM, this exon is preferentially included compared to controls and may act as a compensatory mechanism to increase the abundance of nuclear MBNL. However, the presence of this sequence decreases the splicing activity of MBNLs and that may be related to differences in MBNL homotypic interactions (386, 412).

Additional DM Pathomechanisms

RNA missplicing is a global phenomenon in DM1 and DM2 (302, 425) muscle, and we have largely focused on its role as a driver of disease symptoms. However, the direct role of spliceopathy in disease progression has been called into question due to the common occurrence of DM-relevant missplicing events in a variety of neuromuscular disorders (19). Given these observations, and the fact that many missplicing events are associated with muscle degeneration-regeneration (285), aspects of spliceopathy may be secondary to muscle pathology rather than a driver of disease. Furthermore, additional molecular pathomechanisms are clearly present. For example, bidirectional transcription of CTG^{exp} alleles results in the production of CUG-CAG repeat structures and associated downregulation of endogenous CUG/CAG containing transcripts via an RNAi-like pathway in fly models (453). It is possible similar mechanisms occur in DM-affected tissues, as *SIX5* antisense transcripts can extend through CTG repeats (74). However, the expression

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of *DMPK* antisense transcripts and the abundance of antisense RNA foci appear to be very low (262). Another class of regulatory RNAs, namely miRNAs, are also disrupted in DM *Drosophila* models, with levels of important myogenic miRNAs such as miR-1 and miR-7 reduced and conserved in DM-patient-derived fibroblast and myoblast cell lines with associated increases in several downstream target RNAs (111). In this study, expression of CUG^{exp} RNAs was sufficient to cause these changes and was linked to alterations in *Drosophila* Mbl availability and control of miR-1 and miR-7 biogenesis (111). Interestingly, while miRNA networks are also misregulated in DM1 heart, these alterations are not linked to MBNL loss- or CELF gain-of-function, but rather dysregulation of MEF2 transcriptional networks (187). Heart-specific CUG^{exp} RNA overexpression in the EpA960 mouse model is sufficient to induce these changes, MEF2A protein levels are significantly reduced in DM1 patient heart tissue, and exogenous expression of MEF2C in a DM1 cardiac cell model rescues MEF2 associated miRNA and mRNA expression (187). In addition to MEF2-transcriptional networks, other transcriptional regulators are affected in DM1 mouse models. For example, the abundance and nuclear localization of NFATc1 is increased in *HSA*^{LR} muscle and is coincident with increased levels of transcriptional targets (324). Interestingly, calcineurin (a phosphatase that increases the activity of NFATc1) is also increased in the *HSA*^{LR} mouse model as well as DMD muscle and is thought to act as a compensatory response to calcium mishomeostasis in both (324).

The distinction between RNA and protein toxicity is blurred in the case of another emerging DM-associated pathology, RAN translation. RAN translation was originally identified in the neurological disease SCA type 8 (SCA8) and a mouse model of this disease (463), but is increasingly appreciated in several microsatellite disorders including FXTAS, HD, and *C9orf72*-linked ALS/FTD (78, 463). In the case of HD-associated RAN peptides, a direct toxic role has been revealed using glial and neuronal cell models (22). Furthermore, antisense transcription across *C9orf72*-linked G_4C_2-repeat results in the accumulation of G_2C_4-associated RAN proteins in patient brain that have been demonstrated to exert toxicity in cell model systems (464). In the context of DM1, polyglutamine RAN proteins have been identified in the muscle of DMSXL and DM55 mouse models as well as in patient myoblast cell lines (463). Since, bidirectional transcription can occur across CUG^{exp} tracts in DM1, both CUG and CAG RNAs might escape into the cytoplasm resulting in the synthesis of up to six RAN peptides. For DM2, the identification of RAN proteins is most prevalent in CNS tissues and has not yet been demonstrated in muscle tissues.

Therapeutic Interventions

The efficacy of therapeutic approaches requires the development of measurable outcomes that must be carefully defined. Studies in the past 25 years have linked some DM disease features to specific RNA processing events (e.g., myotonia and insulin insensitivity caused by CLCN1 and INSR missplicing, respectively). These splicing biomarkers are important tools to determine the therapeutic success in mouse models, but the requirement for biopsies from affected tissues undermines their feasibility to evaluate treatment efficacy in clinical trials. In contrast, less invasive approaches can be used to monitor muscle strength and myotonia. Using newly developed equipment to monitor muscle strength, a longitudinal study in the DMSXL mouse has identified outcome measures for preclinical assessments

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(90). Likewise, myotonia can be monitored qualitatively through grip and percussion tests or more quantitatively using EMG. Altogether, these outcome measures have provided reasonable avenues to develop and evaluate multiple strategies for targeted intervention.

While the ideal therapy for DM would be the correction of expanded repeats to a nonpathogenic size, this approach requires further advancements in targeted gene editing as well as a change in societal attitudes toward the use of these technologies in a clinical setting. Alternative strategies have been proposed, including the use of ASOs that target CUG^{exp} transcripts and prevent MBNL sequestration or induce transcript degradation. This approach has been successful in reverting phenotypes in animal models, and holds great promise in clinical trials. Several other avenues have been explored, including the use of small molecules designed to bind repeats and release MBNL or block *DMPK* transcription. In this section, we will discuss these and other potential therapeutic interventions for DM, including their technological limitations and unresolved questions.

Gene editing

Correction of the expanded repeat in the *DMPK* or *CNBP* genes to a nonpathogenic size has been a major objective since the discovery of the mutations underlying DM1 and DM2 (47, 150, 231, 241). For many years, this site-specific modification in the human genome remained technically challenging, mostly due to the substantial complexity and inefficiency of gene-editing approaches. Recent additions to the genome engineering toolbox such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the revolutionary CRISPR/Cas system have provided efficient strategies to accomplish the goal of targeted gene editing (Fig. 14) (93). Currently in Phase II clinical trials, ZFNs were used to KO the CCR5 receptor from autologous hematopoietic stem cells, making them resistant to HIV (395). Similarly, the CRISPR/Cas system has been recently used in a mouse model of DMD, providing a successful approach to remove the mutant exon 23 from the *DMD* gene, producing an in-frame mRNA and a truncated but functional DMD protein (387).

In DM, TALENs have been used to correct patient-derived stem cells by insertion of a polyadenylation signal upstream of the CTG repeats (125, 448). While this strategy does not remove the repeats from the *DMPK* gene, it results in the premature termination of *DMPK* transcription, preventing the production of mutant transcripts that accumulate in RNA foci, and restoring normal splicing patterns in neural progenitor cells. Similarly, expanded CTG repeats can be corrected to a nonpathogenic size or the *DMPK* gene can be knocked out, despite the existence of conflicting reports regarding the effects of *Dmpk* ablation in mice (62, 327).

A recurrent concern with any gene editing strategy is the potential for off-target, or nonspecific, DNA cleavage. To enhance specificity, Cas9 nucleases, which alone are capable of generating a DNA double-stranded break, can be replaced by a pair of nickases, enzymes that can only cut one of the DNA strands (364). As a result, the generation of a double-stranded break now requires the simultaneous and proximal binding of two different nickases, which in turn dramatically reduces the occurrence of off-target DNA cleavage events. Additionally, these nucleases can be engineered for enhanced specificity.

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For example, the widely used Cas9 nuclease from *Staphylococcus pyogenes* (SpCas9) has been modified to alter some hydrogen bond-forming amino acid residues resulting in a Cas9 enzyme with nearly undetectable off-target effects (198).

The largest disadvantage of gene-editing applications for DM1 and DM2 is the multisystemic nature of these diseases. Systemic delivery is very challenging and the idea of correcting all affected tissues is not viable with the current technological repertoire. Local delivery of nucleases to skeletal muscle is possible with adeno-associated virus (AAV) vectors but the cargo is limited to ~4.5 kb so AAVs are not suitable to deliver the relatively large (~4.2 kb) SpCas9 and its guide RNA. As an alternative, smaller, albeit less active, nucleases have been utilized, including the ~3 kb *Staphylococcus aureus* Cas9 (SaCas) that is currently being used in multiple preclinical studies (318, 387).

Antisense oligonucleotides

Currently, ASO technology is the most promising therapeutic avenue for DM. ASOs are short nucleic acid fragments whose sequence is complementary to a target RNA of interest. Depending on the specific chemistry used, ASOs can bind to their RNA target and sterically prevent the association of transacting factors, such as MBNL, or bind and activate targeted degradation of mutant transcripts. The first scenario relies on ASOs such as phosphorodiamidate morpholino oligomers or all-lock-nucleic acid oligomers, which are composed of a short stretch of pure CA(G)G repeats that bind to expanded C(C)UG transcripts without triggering RNA degradation. This enables the release of sequestered MBNL proteins, restoring their function within the cell (439,443). In the second scenario, chimeric ASOs have been developed including DNA gapmers, which consist of 7 to 10 phosphorothioate DNA nucleotides flanked by modified RNA bases. Upon entry into the cell nucleus, these ASOs bind to their target RNA sequences and the subsequent DNA:RNA hybrid is recognized by endogenous RNase H resulting in the degradation of the RNA portion of the duplex (220). Because C(C)UG^{exp} RNAs are largely retained in the nucleus, this increases the potency of therapeutic compounds that require endogenous intranuclear factors such as RNase H. In fact, variations in many factors such as C(C)UG^{exp} repeat length, C(C)UG^{exp} expression level, and available intranuclear MBNL produce a dynamic environment that has important implications for therapeutic intervention (401). For example, under conditions of C(C)UG^{exp} RNA excess relative to MBNL, increased MBNL occupancy within RNA foci may reduce the movement of foci-associated MBNL into the nucleoplasm resulting in increased molecular crowding with implications in preventing the availability of C(C)UG^{exp} to small molecules or ASOs (386,401). As hundreds of nuclei are present in muscle fibers, it is possible that different disease progression states exist in distinct nuclei within a single muscle cell. Beyond targeting toxic C(C)UG^{exp} RNAs, ASOs can modulate downstream effectors by targeting RNA processing events underlying specific DM symptoms, such as *CLCN1* missplicing (201). Similar approaches have been successfully used to promote exon skipping in Becker and Duchenne muscular dystrophies (1) or enhanced *SMN2* exon 7 inclusion in spinal muscular atrophy (169). Intramuscular delivery of ASOs has been shown to be effective (220), despite the fact that membrane integrity is not significantly compromised in DM (133).

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Systemic distribution of ASOs is challenging, but studies have shown it can be improved by peptide-linkage strategies (229) and liposomal delivery followed by ultrasound exposure (201). Changes in ASO chemical composition affect tissue permeability, distribution, stability, affinity for repeats, and the mechanism of action. For instance, modified 2'-O-methoxyethyl (MOE) flanking gapmers increase ASO bioavailability, resulting in a remarkable reversion of ~85% of the splicing changes in the HSA^{LR} mouse model (438).

Following their marked success in preclinical studies, ASOs designed to degrade mutant *DMPK* transcripts entered a Phase I/II clinical trial in December 2014. In early 2017, this trial was discontinued due to inadequate efficacy in muscle tissue although safety was not an issue. Modifications in ASO composition are promising strategies to overcome this issue and future preclinical studies and therapeutic trials will inform us regarding the success of these structural changes, including their impact on efficacy, tissue distribution, and long-term turnover. Importantly, long-term studies are required to address several safety issues concerning ASOs, including their specificity and toxicity.

The concept of toxicity is limited by the assay and thus ASO off-target effects must be carefully monitored using different experimental approaches. For example, the current generation of DNA gapmers for DM1 is designed to preferentially degrade mutant DMPK transcripts, due to their nuclear retention and RNase H susceptibility, but transcript levels from the normal allele are also affected and depletion of this kinase may adversely affect the regulation of its phosphorylation targets. For DM2, *Cnbp* is an essential gene in mice so gapmers targeting human CNBP exonic regions may lead to deleterious consequences. An alternative is to target the $CCUG^{exp}$ region directly, but other genes containing CCUG repeats may be affected so global gene expression studies should also be performed to address this issue. Additionally, the downstream consequences of morpholinos aimed to release MBNL proteins from toxic transcripts must be evaluated. Once free from MBNL, toxic transcripts might be exported to the cytoplasm and trigger RAN translation or RNAi pathway alterations (111,205,208). Despite their occurrence in DM, the specific contribution of these pathways for DM disease pathogenesis remains unclear.

Small molecules

The knowledge gathered from structural studies on expanded C(C)UG RNAs and their interaction with MBNL proteins has paved another avenue for therapeutic intervention: the use of small molecules that displace or sterically prevent MBNL binding. Designed to specifically bind C(C)UG repeats, small molecules are promising therapeutic candidates due to their lower production costs and potential for increased tissue permeability. To date, several compounds have been proposed, including Ligand 1 (145), Hoechst 33258 (314), lomofungin and dilomofungin (167), and others (71, 72, 293). While the majority of these compounds specifically bind to CUG repeats, some target CCUG expansions as well (72, 228).

Another promising class of small molecules consists of transcriptional inhibitors that intercalate into GC-rich DNA, arresting elongating RNA polymerase II (118). These compounds are mostly chemotherapeutics with high affinity and specificity for CUG repeats at low dosages. For example, actinomycin D (ActD), an FDA-approved drug used to

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treat cancers at dosages between 0.015 and 0.045 mg/kg, was deployed to reduce CUG expression at a human equivalent dosage of 0.002 mg/kg and rescue splicing at 0.02 mg/kg in the *HSA*^{LR} mouse model (366). Nevertheless, the ability of ActD to induce global transcriptional inhibition is a potential drawback and its long-term side effects are unknown. Currently, our repertoire of small molecules is expanding, primarily supported by high-throughput screens that address the impact of these compounds on RNA foci formation, splicing rescue, MBNL1 and CELF1 localization and function (195).

Additional strategies

Several additional approaches have been proposed as potential therapeutic interventions for DM. For example, the utilization of mexiletine has been shown to ameliorate myotonia (234) and the use of steroids such as dehydroepiandrosterone (DHEA) to treat muscle weakness (299). Exercise has been suggested as another therapy (196), as well as the implantation of automatic defibrillators to prevent sudden death, despite the risks of exposing DM patients to generalized anesthesia (32, 426).

At the cellular and molecular levels, multiple additional strategies have also been explored. Artificial site-specific RNA endonucleases have been used to degrade expanded CUG transcripts (458). In patient-derived cells, engineered human U7 small nuclear RNAs containing a CAG repeat were utilized to selectively degrade mutant *DMPK* transcripts (116). Complete *DMPK* knockdown has also been suggested (217, 372), as well as *MBNL1* overexpression, despite the potential detrimental effects of these strategies in humans (190).

Conclusion

In this review, we have summarized key concepts of skeletal myopathy in DM. However, DM1, DM2, and CDM are multisystemic diseases and require a multifaceted approach to pinpoint the key drivers of pathology in the various affected tissues. We believe the use of increasingly sophisticated animal models will support these studies. Current generations of DM modeling studies have revealed recurrent themes in DM pathomechanisms, but no animal model recapitulates the complete spectrum of DM disease. While differences between mouse and humans may prevent this, attempts to generate these models have largely been stifled by technical barriers. First, the generation of expanded DNA repeats is limited by the capacity of *E. coli* to harbor large expansions and poses a barrier to traditional cloning strategies (33). Techniques such as rolling circle amplification have been utilized to circumvent this, allowing for the generation of CTG repeats well beyond the CDM threshold of ~1000 (286). While theoretically possible, it remains to be seen if similar successes can be achieved with CCTG expansions. Even with DNA templates of the appropriate length, introduction into the mouse genome is challenging particularly for targeted knockins. The use of traditional mouse ESC targeting strategies have not yielded models with large expansions possibly due to toxicity of these lengthy repeats in these cell populations or the inability of endogenous mouse loci to harbor large expansions. To date, CTG$^{<100}$ is the largest repeat knocked into the mouse *Dmpk* locus (417). The use of newer technologies, such as CRISPR/Cas9, may provide a valuable resource in the generation of a new generation of knockin models. Furthermore, while many current transgenic models

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of DM are available, few insertions have been mapped and will be important to ensure off-target effects are not contributing to phenotypes observed in these animals. Given CRISPR/Cas9 is also susceptible to off target effects, careful mapping and characterization of mutations should be performed in conjunction with efforts to phenotype models.

Another largely unanswered question in the field is the mechanistic basis of congenital DM. One key distinction between DM1 and CDM is the presence of highly expanded CTG^{exp} repeats throughout embryonic development in CDM due to inheritance of large alleles. Similar to adult-onset DM1 where RNA alternative processing is linked to disease manifestations, the presence of highly expanded CUG^{exp} RNAs during embryonic development would be expected to affect RNA isoform transitions important for the tissue morphogenesis (186). In agreement with the RNA toxicity model holding true during tissue development, CUG^{exp} RNA foci are highly abundant in embryonic and fetal cardiac, skeletal muscle, and brain tissues expressing CUG^{exp} RNA (262). These data suggest MBNL loss-of-function contributes to CDM disease, a hypothesis supported by our recent findings of congenital myopathy in mouse *Mbnl* KO models (397). Beyond inherent toxicity throughout embryonic development, the dynamics of repeat instability may differ in developing tissues compared to postmitotic tissues (255). Along with these, the lack of congenital forms of DM2 suggests fundamental differences in the ability of *CNBP*-linked $CCTG^{exp}$ mechanisms to elicit developmental defects. Differences in the spatiotemporal expression and steady-state transcript levels of $CCUG^{exp}$ versus CUG^{exp} containing transcripts may underlie this difference, but this question warrants further investigation.

Acknowledgements

The authors thank J. Bubenik for reviewing the manuscript and members of E. Wang's lab for lending a hand. Research studies performed in the Swanson lab are supported by grants from the NIH (NS058901, NS98819) and the MDA (RG480539). The authors also thank the Myotonic Dystrophy Foundation and Wyck Foundation for support to Ł.J. Sznajder.

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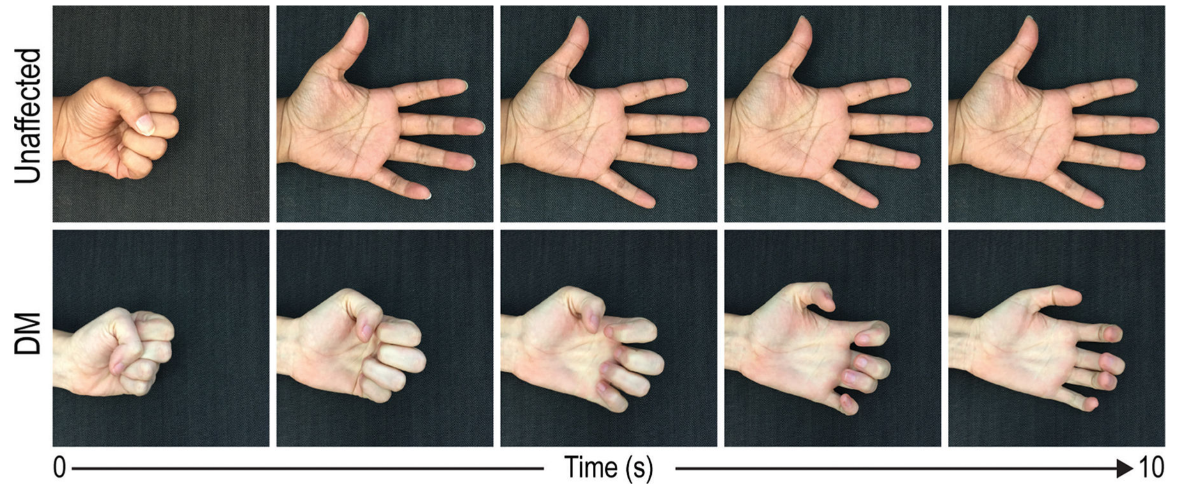

Figure 1.
Myotonia is a characteristic skeletal muscle feature of DM patients. In unaffected individuals, grip relaxation is unencumbered and accompanied by muscle repolarization to resting potential (upper panels). For DM patients, loss of ion homeostasis results in delayed relaxation (lower panels).

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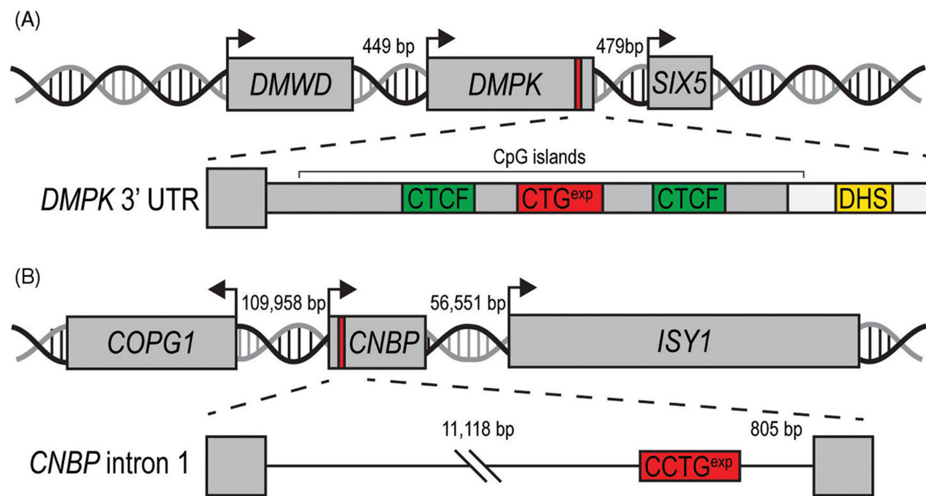

Figure 2.

DM1- and DM2-associated gene loci. (A) The *DMPK* CTG^{exp} (red box) is located in the 3' UTR and is adjacent to two closely neighboring genes, *DMWD* and *SIX5* (arrows indicate transcription start sites). CTCF binding sites (green boxes) flank the CTG^{exp} along with a downstream DNase hypersensitivity site (DHS, yellow box). These elements may regulate the epigenetic features of this locus. (B) The DM2-associated CCTG^{exp} (red box) is located in the first intron of *CNBP*. Neighboring genes are distal to this locus and may not be affected by this microsatellite expansion.

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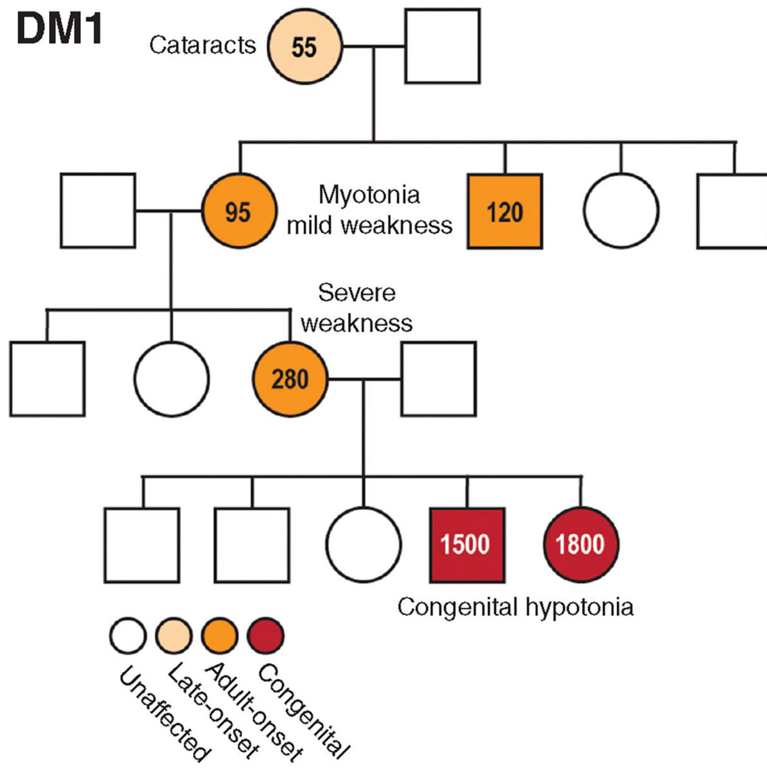

Figure 3.
DM1 pedigree highlights genetic anticipation. Hypothetical pedigree of a DM1 family with males (boxes) and females (circles) and mutant allele CTG repeat lengths indicated.

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Disease	Subtype	Repeat #	Skeletal muscle	Other tissues
DM1 *DMPK* CTG^{exp}	CDM	> 1000	Hypotonia, muscle immaturity, respiratory distress, polyhydraminos, dysphagia, talipes	Heart defects, intellectual disabilitiy
	Juvenile-onset	50 to 1000	Myotonia (grip, percussion, electrical), weakness, distal muscle wasting, insulin resistance	Heart defects, hypersomnia, cataracts, endocrine dysfunction
	Adult-onset	50 to 1000		
	Late-onset	50 to 1000		
	Premutation	38 to 49	Normal	Normal
	Normal	5 to 37	Normal	Normal
DM2 *CNBP* CCTG^{exp}	Affected	> 75 to 11,000	Myotonia (grip, percussion), proximal muscle wasting, muscle hypertrophy, myalgia	Heart defects, cataracts, endocrine dysfunction
	Normal	10 to 35	Normal	Normal

Figure 4.

Clinical manifestations and disease stages in DM1, DM2, and CDM. In DM1, a variety of clinically defined subtypes are listed along with associated symptoms. While juvenile-, adult- and late-onset DM1 are all listed with 50 to 1000 repeats, earlier age-of-onset and exacerbated disease severity typically correlate with increased CTG^{exp} size in DM1. This correlation is not as marked for DM2.

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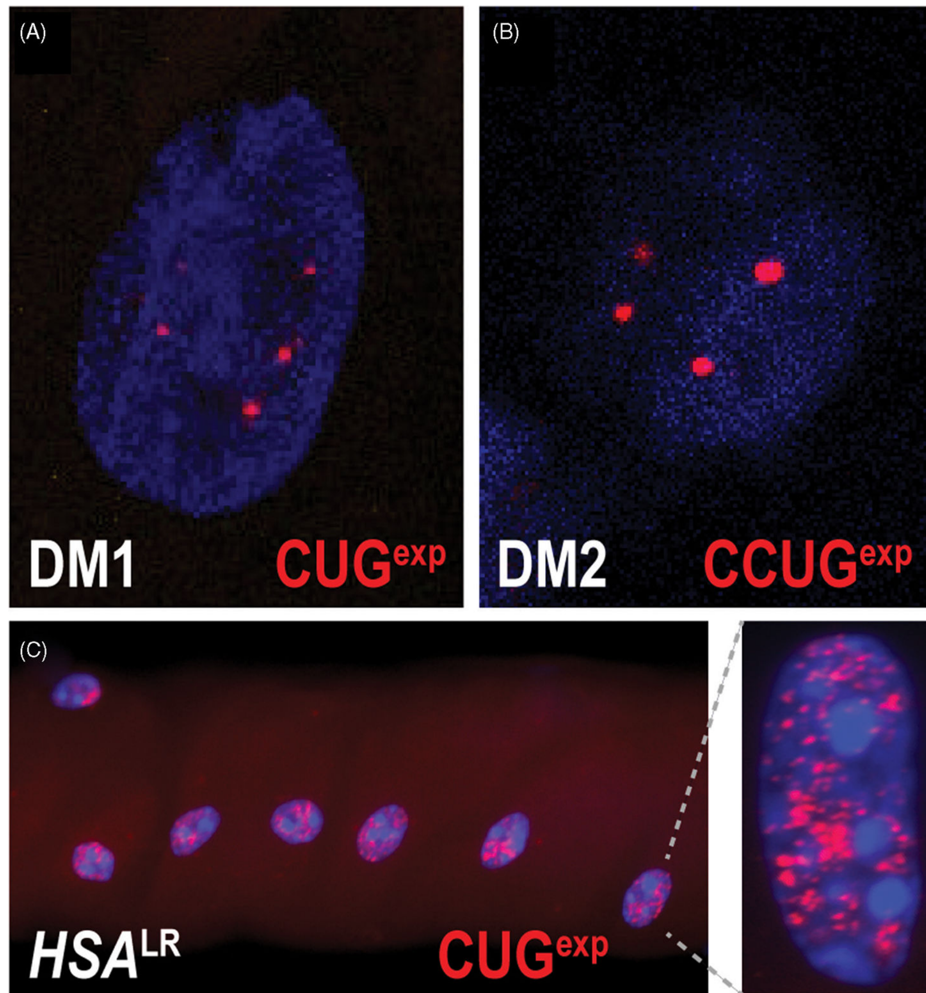

Figure 5.
RNA foci in myotonic dystrophy. ((A) and (B)) Fluorescently labelled $(CAG)^{10}$ or $(CAGG)^{10}$ oligonucleotide probes hybridize to DMPK CUG^{exp} transcripts in DM1 (A) or CNBP $CCUG^{exp}$ in DM2 (B), and reveal a punctate intranuclear staining pattern. These observations support the hypothesis that these mutant RNA transcripts are blocked for nucleocytoplasmic export and could exert toxicity in the nucleus. (C) Nuclear foci are abundant in myofibers isolated from the *HSA*^{LR} mouse DM1 model.

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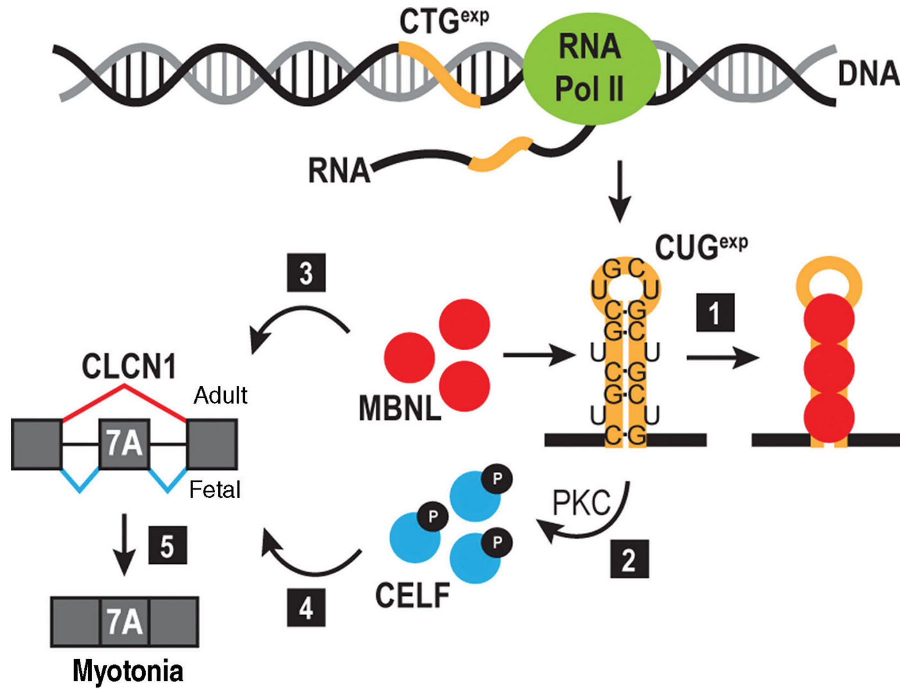

Figure 6.

RNA toxicity model. Expression of the *DMPK* 3' UTR CTG^{exp} (orange line) produces a CUG^{exp} RNA that sequesters MBNL proteins (red circles) (1) and triggers protein kinase C (PKC)-mediated CELF1 hyperphosphorylation (2) leading to an increase in its steady-state level. CELF and MBNL are antagonistic regulators of alternative splicing with MBNL promoting adult (3), and CELF favoring fetal (4), splicing isoforms. MBNL sequestration by CUG^{exp}, in addition to CELF stabilization, leads to an imbalance in alternative splicing and emergence of fetal isoforms in adult tissues. In DM, this cascade leads to inclusion of exon 7A in CLCN1 mRNA, generating a fetal transcript that is degraded by nonsense-mediated decay. The absence of CLCN1 in the muscle membrane results in myotonia (5).

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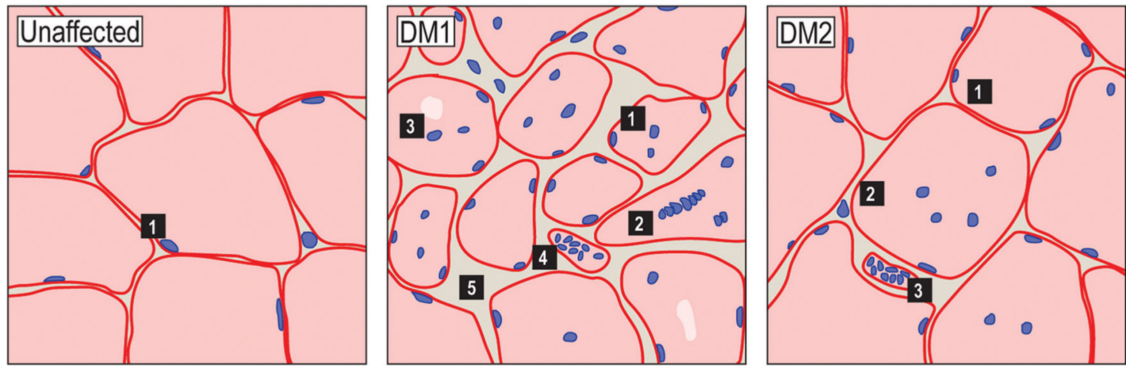

Figure 7.

Histological features of DM1 and DM2 skeletal muscle. Schematic representations of H&E-stained skeletal muscle cross-sections from unaffected (left), DM1 (center), and DM2 (right) patients depicting common histological features (images available at http://neuromuscular.wustl.edu/pathol/). Typically, myofibers are uniform in size and have subsarcolemmal myonuclei (left panel). In DM1, histopathological features include central myonuclei, myofiber size variability, pyknotic nuclear clumps and fibrosis. Other features include type I fiber atrophy, irregular nuclei shape, and acid phosphatase stained granules and several of these features roughly correlate with disease severity and progression. In DM2, these histopathological features are generally less pronounced and may include some variability in fiber size, internal myonuclei, and pyknotic nuclear clumps. Acid phosphatase positive granules are also observed in DM2.

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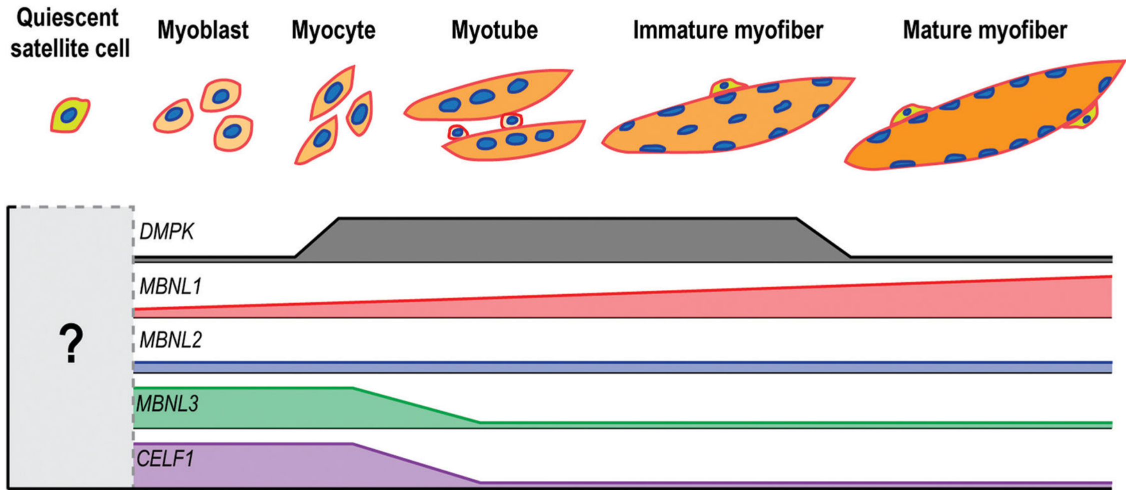

Figure 8.

Expression patterns of DM-associated transcripts throughout myogenesis. As muscle precursor cells differentiate and mature into adult myofibers, the expression of *DMPK* (grey) increases transiently. *MBNL1* (red) levels increase steadily as muscle develops while *MBNL2* (blue) levels remain relatively constant. Both *MBNL3* (green) and *CELF1* (purple) are associated with early muscle precursors and other embryonic cell populations. The relative expression level of these genes in quiescent satellite cells is currently unknown. While *CNBP* (not shown) is highly expressed in proliferative cell populations, its relative expression in various myogenic cells is unclear.

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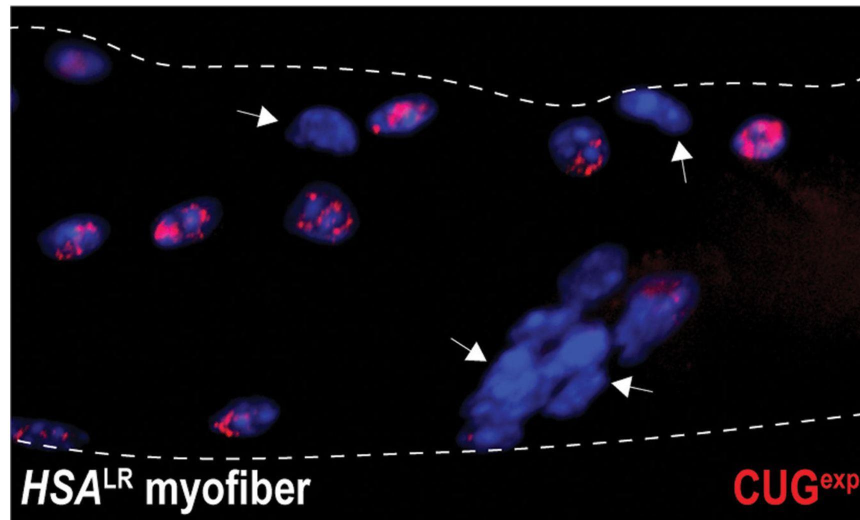

Figure 9.

RNA foci in *HSA*^{LR} myofibers. A nonuniform distribution of RNA foci-positive (red) and negative (white arrows) nuclei (blue, DAPI) is present in *HSA*^{LR} myofibers. Foci-negative nuclei are likely satellite cells, subjunctional myonuclei, or nuclei from other myofiber-associated cells. This is the expression pattern generated by the *HSA* promoter, so expression of DMPK CUG^{exp} RNAs in these nuclei may contribute to disease progression in DM1 patients.

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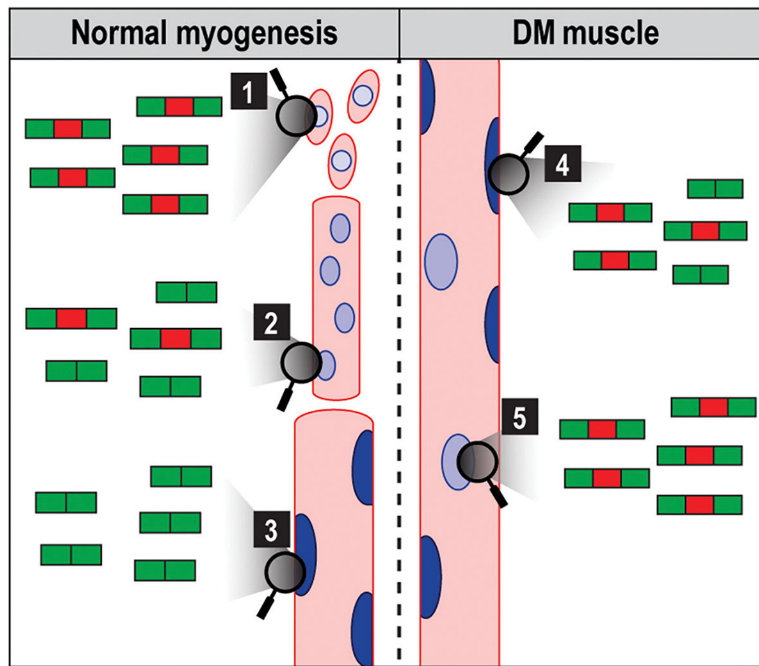

Figure 10.

RNA splicing in unaffected and DM muscles. In unaffected adults, C(C)UG repeat number is in the nonpathogenic range and adult/mature RNA isoforms (red exon exclusion) are expressed (3). During injury-induced regeneration, fetal RNA isoform (red exon inclusion (1) and (2)) expression patterns are recapitulated. In DM, $C(C)UG^{exp}$ RNA expression inhibits MBNL splicing activity by sequestration leading to fetal/immature isoform reexpression in mature myofibers (4), which is also accompanied by elevated regeneration indicated by centralized myonuclei (5).

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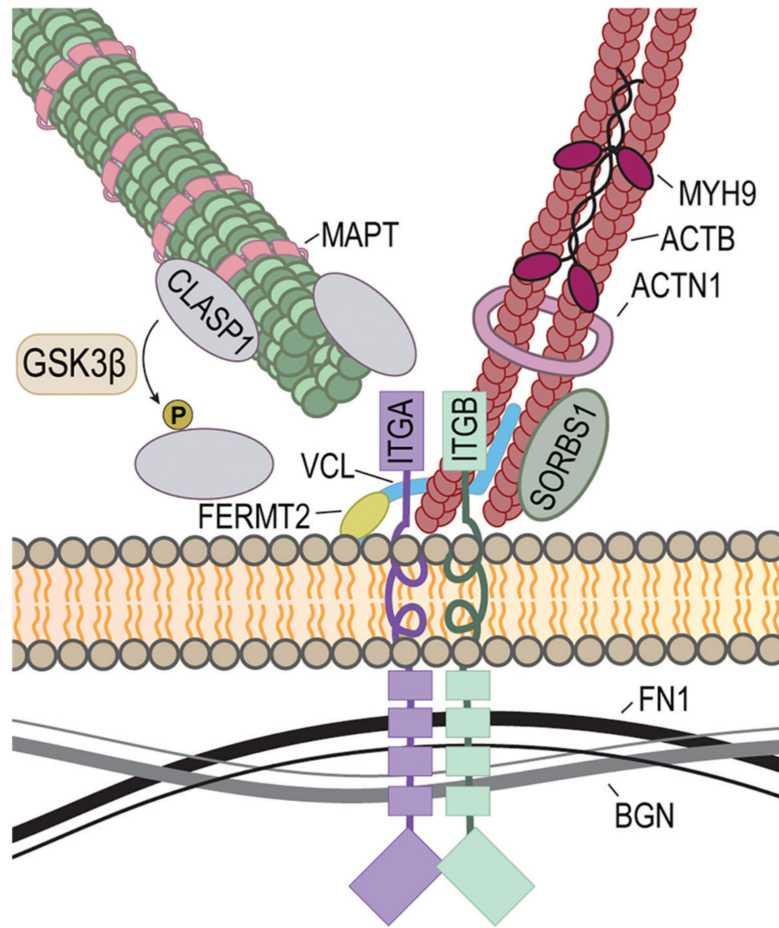

Figure 11.
DM-associated components of focal adhesions. A schematic of a focal adhesion is shown along with some associated components implicated in DM.

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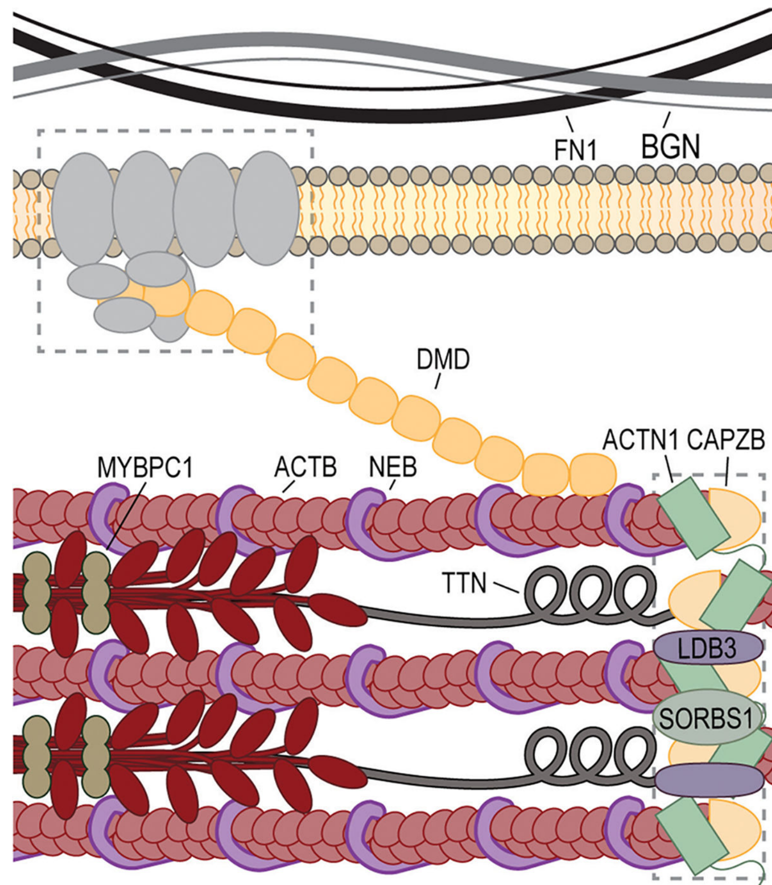

Figure 12.
DM-associated contractile and structural proteins. A schematic of a sarcomere is shown along with the DMD-mediated link to the sarcolemma. Gray boxes are shown outlining the dystrophin-associated glycoprotein complex (left) and the muscle Z-line (right).

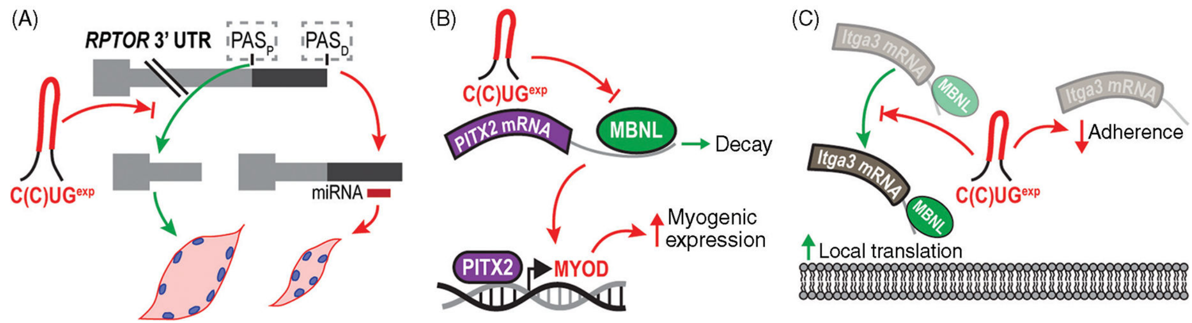

Figure 13.

Additional RNA processing events implicated in DM. (A) *RPTOR* polyadenylation site (PAS) selection (PAS_P, proximal PAS; PAS_D, distal PAS) is altered in DM1 by CUG^{exp} RNA and perhaps $CCUG^{exp}$ RNAs (red hairpin) in DM2. Increased PAS_D utilization may contribute to muscle wasting in DM because the increased 3' UTR length allows regulation by miRNAs (red box) (24). (B) MBNL1 contributes to *PITX2* mRNA (purple box) decay (green arrow), and $C(C)UG^{exp}$-associated blocking of MBNL increases PITX2-mediated myogenic gene expression (244). (C) MBNL2/MLP1 has also been proposed to regulate ITGA3 mRNA localization to focal adhesions, presumably to allow local translation at these sites. Disruption of this activity in DM has been proposed to affect cell adherence (4).

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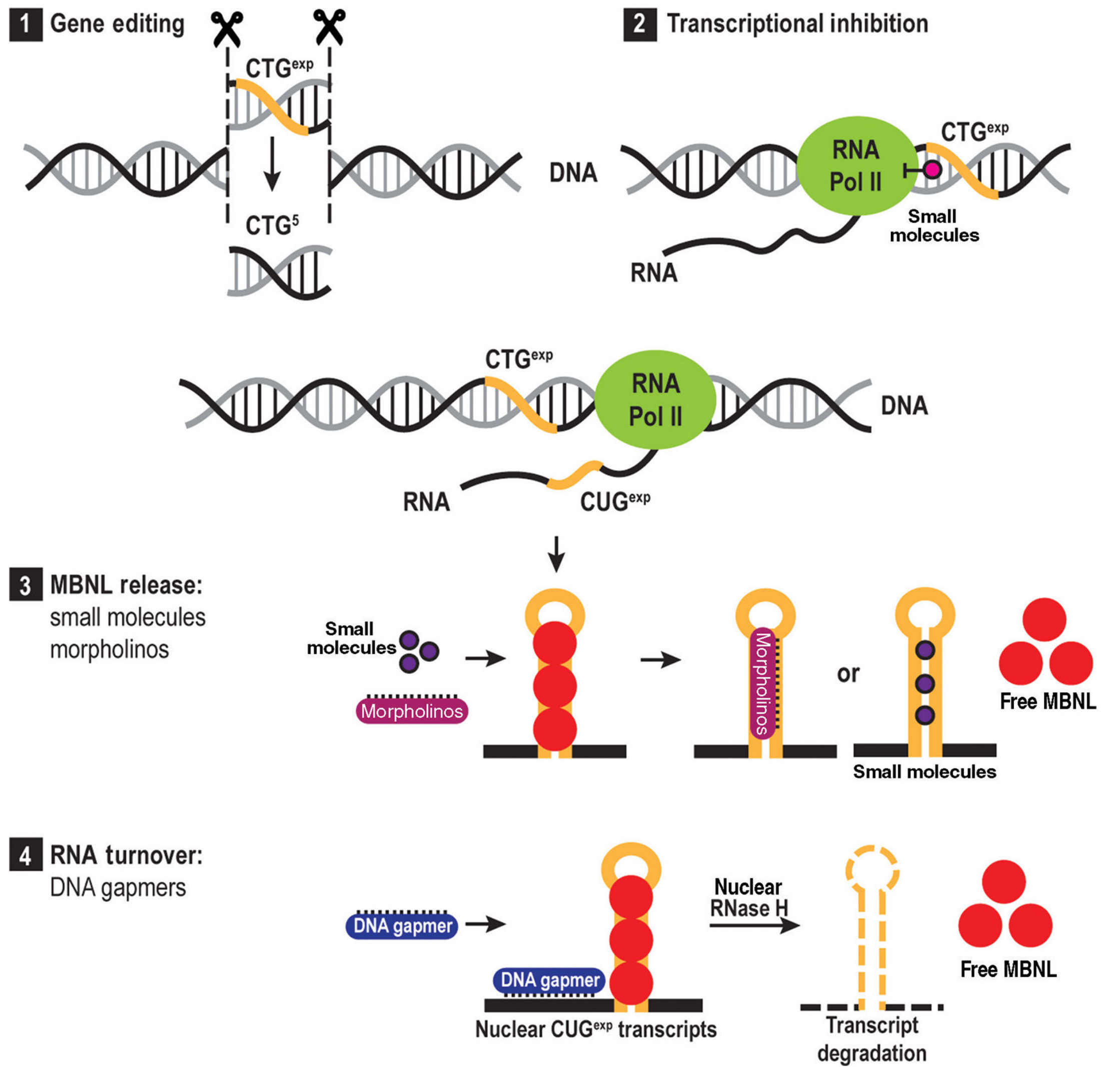

Figure 14.

Therapeutic interventions. Proposed avenues for therapeutic intervention in DM, including: (1) gene editing of the expanded repeats to a nonpathogenic size; (2) use of small molecules that intercalate into GC-rich DNA and arrest the elongating RNA polymerase II; (3) use of small molecules or morpholinos that displace or sterically inhibit MBNL binding; (4) use of DNA antisense oligonucleotide (ASO) gapmers that bind to mutant transcripts and trigger their degradation by RNase H.

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Table 1

DM Mouse Models

Mouse model	Rationale	Features and contributions	Limitations	References
Dmpk knockout	*DMPK* haploinsufficiency in DM1.	Established that DM1 is not primarily caused by *DMPK* haploinsufficiency.	Conflicting reports on impacts of *Dmpk* ablation in mice.	Reddy et al., 1996. Carrell et al., 2016.
Six5 knockout	*SIX5* haploinsufficiency in DM1.	Established that DM1 is not caused by *SIX5* haploinsufficiency.	Animals develop nuclear cataracts, different from the subcapsular cataracts in DM1.	Klesert et al., 2000. Sarkar et al., 2000. Wakimoto et al., 2002. Sarkar et al., 2004.
Cnbp knockout	*CNBP* haploinsufficiency in DM2.	Heterozygous mice present myotonia, cardiac conduction defects, cataracts, and myopathic features.	Conflicting reports of CNBP downregulation in DM2.	Chen et al. 2003. Chen et al., 2007. Margolis et al., 2006. Raheem et al., 2010.
Mbnl1 knockout	*Mbnl1* loss-of-function.	Presents with myotonia, aberrant splicing, centralized myonuclei, and subcapsular cataracts.	Not a C(C)TG^{exp} model so may not address some repeat-induced pathomechanisms including RAN translation, RNAi and CELF stabilization. Consitutive loss of both MBNL1 and 2 is embryonic lethal.	Kanadia et al., 2003. Lee et al., 2013.
Mbnl2 knockout	*Mbnl2* loss-of-function	Develops DM-relevant learning deficits and REM sleep disturbances		Charizanis et al., 2012.
Mbnl3 knockout	*Mbnl3* loss-of-function.	Absence of nuclear isoform impairs muscle regeneration		Poulos et al., 2013.
CELF transgenic	Overexpression of CELF in skeletal muscle or heart.	Presents molecular, histological and physiological features of DM1.	Premature lethality. Limited applications for therapeutic development.	Timchenko et al., 2004. Ward et al., 2010. Koshelev et al., 2010.
Dmt	First DM1 transgenic model. Transgene comprises ~ 162 CTG repeats and ~750 bp of flanking DNA from the *DMPK* 3' UTR without any promoter element or coding sequence.	Occurrence of somatic and intergenerational instability.	Absence of promoter elements prevents transgene expression, limiting the applicability of this model to studies on repeat instability.	Monckton et al., 1997. Fortune et al., 2000.
HSA^{LR}	Transgenic overexpression of CTG^{250} within the human skeletal actin (*HSA*) 3' UTR.	Established the RNA gain-of-function mechanism underlying DM1. Shows RNA foci and splicing alterations. Widely used for therapeutic development.	Transgene is expressed only in postdevelopmental skeletal muscle tissue at levels much higher than the endogenous *Dmpk* gene. Absence of muscle weakness or wasting. No effect on CELF levels.	Mankodi et al., 2000.
DM300	Transgenic line carrying a 45 kb region from the DM1 locus including a mutant *DMPK* gene with ~300 CTG repeats.	Occurrence of somatic and intergenerational repeat instability. Myotonia and histological changes.	Variable expression levels. Mild phenotype.	Seznec et al., 2000. Seznec et al., 2001.
DMSXL	Transgenic line derived from the DM300 model. Contains > 1000 CTG repeats.	Splicing changes in the central nervous system and skeletal muscle of homozygous mice.	Derived from a single founder. Low *DMPK* expression. Requires homozygosity to model a dominant disease.	Gomes-Pereira et al., 2007. Huguet et al., 2012.
EpA960	Tissue-specific overexpression of 960 interrupted CTG repeats within exon 15 of *DMPK*.	Suggested that CELF1 upregulation is dependent on the *DMPK* genomic context.	Interrupted repeat tract. Heart-specific model displayed premature death.	Wang et al., 2007. Orengo et al., 2008.

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Mouse model	Rationale	Features and contributions	Limitations	References
TRE-EGFP-CTG^5 and CTG^{200}	Transgenic line with inducible expression of *DMPK* 3' UTR carrying 5 or 200 CTG repeats.	Dox-induced transgene activation leads to myotonia and cardiac defects. Dox withdrawal reverts these phenotypes.	Overexpression of a nonpathogenic repeat, CTG^5, results in DM1 features.	Mahadevan et al., 2006.
Humanized *Dmpk* CTG^{84} knock-in	Transgenic line in which the genomic fragment between exons 13–15 from the endogenous *Dmpk* gene was replaced by the orthologous human fragment including 84 CTG repeats.	Somatic instability blocked in an Msh3-deficient background and increased in an Msh6-deficient background.	Absence of published data addressing molecular, histological or physiological phenotypes from the expression of humanized mutant *Dmpk* alleles.	van den Broek et al., 2002.

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Table 2

Missplicing Events Associated with DM Disease Symptoms

Gene	Shift in DM	Protein function	Molecular and physiological consequence	References
CLCN1	Increased inclusion of exon 7a	Major skeletal muscle chloride channel responsible for transmembrane chloride conductance	Nonsense-mediated decay of transcript and loss of CLCN1 protein; myotonia	Mankodi et al., 2002. Charlet et al., 2002. Wheeler et al., 2009.
CACNA1S	Decreased inclusion of exon 29	CaV1.1 calcium voltage-gated calcium channel involved in excitation-contraction coupling	Increase CaV1.1 conductance and voltage sensitivity; in mature muscle fibers enhances electrically evoked Ca^{++} release; muscle weakness	Tang et al., 2012.
SCN5A	Increased inclusion of exon 6a and decreased inclusion of exon 6b	Cardiac sodium channel responsible for cardiomyocyte excitability and normal cardiac-conduction system function	Reduced excitability; heart arrhythmia, cardiac conduction delay	Freyermuth et al. 2016.
BIN1	Decreased inclusion of exon 11	Regulates T-tubule biogenesis	Lack of phosphatidylinositol 5-phosphate-binding and membrane-tubulating activities, altered T-tubules; muscle weakness	Fugier et al. 2011.
DMD	Decreased inclusion of exon 78	Large structural and signaling protein linking the actin cytoskeletal to the extracellular matrix through the DAG complex	The dystrophin C-terminus is switched from an adult structure of a 13 aa beta-sheet into an embryonic 31 aa amphipathic alpha-helix. Abnormal dystrophin activity; muscle weakness and progressive atrophy	Rau et al. 2015.
INSR	Decreased inclusion of exon 11 (increased IR-A isoform)	Insulin receptor that mediates signal transduction involved in glucose storage and handling	Higher affinity for insulin; faster internalization and recycling time; lower signaling capacity	Savkur et al. 2001.
PKM	Increased inclusion of exon 10 (increased PKM2 isoform)	Pyruvate kinase M catalyzes phosphate group transfer reactions from phosphoenolpyruvate to ADP in glycolysis	Allosterically regulated isoform with reduced oxygen and increased glucose consumption; abnormal regulation of glucose metabolism in muscle; compromised glucose homeostasis; contributes to type 1 myofiber atrophy	Gao et al. 2013.
MBNL1, MBNL2	Increased inclusion of exon 54nt, exon 36nt, and exon 95nt	RNA binding proteins that regulate diverse RNA processing activities	Increased nuclear localization and RNA splicing and polyadenylation activity	Sznajder et al. 2016. Kino et al. 2015. Tran et al. 2010. Yuna et al. 2007.