

Chapter 3

Myotonic Dystrophy

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Abstract Myotonic dystrophy (DM) is the most common form of muscular dystrophy in adults, caused by unstable genomic expansions of simple tandem repeats. Myotonic dystrophy type 1 (DM1) results from expansion of a CTG repeat in the 3' untranslated region of *DMPK*. In myotonic dystrophy type 2 (DM2), the expanded repeat is a CCTG tetramer in intron 1 of *CNBP/ZNF9*. The mRNA transcripts containing expanded repeats form ribonuclear inclusions, thereby retained in the nucleus. The mutant RNA gives rise to a toxic gain of function by perturbing splicing factors, resulting in misregulation of alternative pre-mRNA splicing that may underlie the multisystemic symptoms of DM. Although no curative treatment exists, recent advances in basic and translational research and pharmacological approaches provide clues for therapeutic intervention in DM. In this review, we describe the RNA-dominant mechanism in DM and summarize potential therapeutic approaches that address RNA toxicity and current progress toward translational research.

Keywords Trinucleotide • Toxic RNA • Repeat instability • Alternative splicing • Spliceopathy

3.1 Myotonic Dystrophy

Myotonic dystrophy (DM) is the most common type of muscular dystrophy in adults, with a prevalence of 1 per 8,000 individuals [1]. DM is a dominantly inherited multisystemic disorder caused by the expansion of CTG (myotonic dystrophy type 1, DM1) or CCTG (myotonic dystrophy type 2, DM2) repeats [2, 3]. The common molecular basis of DM1 and DM2 is a toxic RNA transcript containing the expanded repeats. DM is the first disease proven to result from by toxic gain of function of the mutant mRNA and is now recognized as an RNA-mediated disease [4]. The clinical expression of both types of DM is quite similar, including myotonia, progressive muscle weakness, cataracts, insulin

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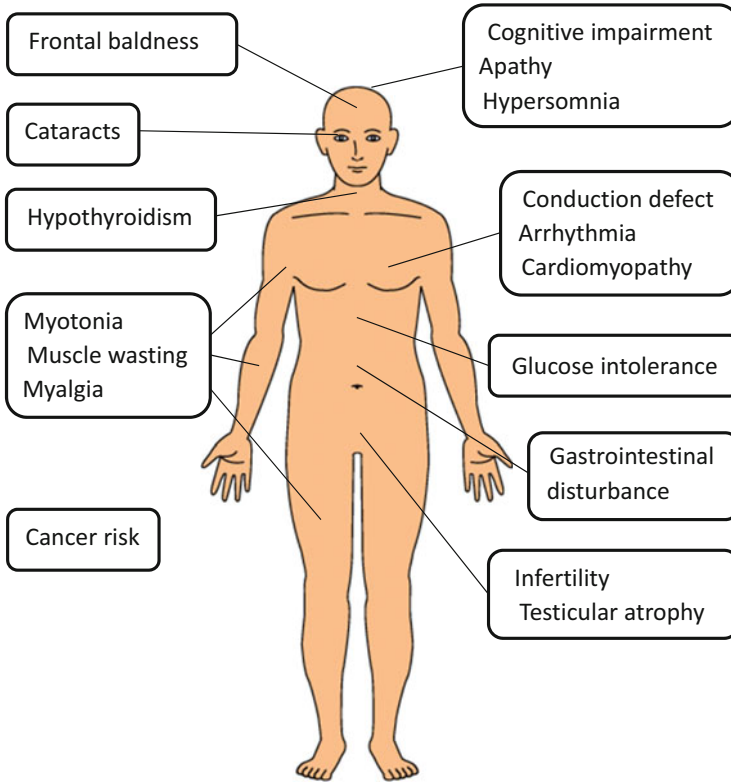


Fig. 3.1 Multisystemic symptoms of DM

resistance, and cardiac conduction defects, although some differences exist between DM1 and DM2 (Fig. 3.1).

3.2 Clinical Features

3.2.1 Adult-Onset DM1

3.2.1.1 Muscle Weakness

The predominant symptom of adult-onset DM1 is facial and distal muscle weakness. Facial weakness is an early feature and atrophy of temporalis and masseter leads to the characteristic facial appearance, so-called hatchet face. Mild ptosis is often seen, but ophthalmoplegia is rare. The neck flexors and sternocleidomastoid muscles are commonly involved. Weakness in the limbs is initially distal, especially finger and wrist flexors and ankle dorsiflexors, leading to difficulty with

opening caps and foot drop. Muscle weakness progresses gradually and proximal limb weakness occurs later in the disease course, leading to loss of ambulation. Swallowing and respiratory muscles are often involved in later disease stages, with resultant difficulty in swallowing and breathing.

3.2.1.2 Myotonia

Myotonia, defined as delayed muscle relaxation following voluntary contraction or mechanical percussion, is caused by abnormal muscle fiber membrane activity [5]. Grip myotonia, which is difficulty in relaxing the grip, is often observed in adult-onset DM1. Improvement in myotonia by repeated contractions is termed “warm-up phenomenon.” Myotonia can be elicited by percussion of the thenar or tongue. Myotonia can be recorded with electromyography (EMG) as bursts of repetitive muscle fiber discharges, giving a characteristic sound called “dive bomber” or “motorcycle.”

3.2.1.3 Cardiac Involvement

Cardiac involvement is the second most frequent cause of death in DM1 [6]. Conduction disturbance is quite common, such as first-degree atrioventricular (AV) block and QRS widening, sometimes progressing to third-degree AV block. Atrial tachyarrhythmia and fatal ventricular tachyarrhythmia can occur. Regular ECG monitoring and early implantation of a pacemaker or cardiac defibrillator are critical for the prevention of sudden unexpected death. In contrast to cardiac arrhythmia, cardiomyopathy is not common, though some DM1 patients suffer from severe dilated cardiomyopathy and heart failure.

3.2.1.4 CNS Involvement

Central nervous system (CNS) involvement is frequently seen in adult-onset DM1. The characteristic neuropsychiatric features are mild cognitive impairment, inertia, apathy, and reduced perception of disease symptoms. Hypersomnia is extremely common even in the absence of respiratory involvement. Polysomnography shows nonobstructive apneas and central hypoventilation. Brain MRI scan frequently shows diffuse white matter lesions, particularly in the frontal or anterior temporal lobes.

3.2.1.5 Endocrine Involvement

Although overt diabetes is not frequent, hyperinsulinemia and insulin resistance are common in adult-onset DM1. Hypothyroidism, dyslipidemia, and male infertility because of testicular atrophy are also seen.

3.2.1.6 Other Multisystemic Symptoms

In addition to striated muscle, smooth muscle is affected in DM1. Gastrointestinal problems, such as diarrhea and constipation, are particularly frequent and may lead to pseudo-obstruction and megacolon. Cholecystitis and gallstones are also common because of increased tone of the gallbladder sphincter. Cataracts (typically iridescent posterior subcapsular forms) develop in most adult-onset DM1 patients and are sometimes the only symptom of very late-onset mild DM1. Early frontal baldness is often seen in male and even in some female DM1 patients. Increased risks of cancers of endometrium, ovary, thyroid, and colon have been reported [7].

3.2.2 Congenital DM1

Congenital DM1 (CDM) is not merely a severe early form of DM1 but rather a distinct clinical phenotype. CDM patients are severely affected at birth with hypotonia and generalized muscle weakness resulting in difficulty of sucking, swallowing, and breathing. Some CDM patients present symptoms even in utero, such as reduced fetal movements and hydramnios in later pregnancy. Talipes and tented upper lip (so-called carp mouth) are also seen in CDM. Despite neonatal intensive support, the mortality from respiratory failure remains high in affected infants. Surviving infants gradually improve in motor function, but nevertheless show delayed motor and mental development. Children with CDM can achieve independent walking, but develop myotonia and progressive muscle weakness in early adulthood. CDM is almost exclusively maternally inherited, even from mothers with mild forms of DM (see Sect. 3.3.2).

3.2.3 DM2

Although DM1 is common in populations of European and Asian descent, most DM2 patients are of northern and eastern European descent. DM2 patients also present myotonia and muscle weakness; however, the weakness typically affects the proximal muscles including the neck, elbow extension, and hip flexors. Significant muscle pain and fatigue are common in DM2. Cardiac conduction defects, cataracts, and insulin resistance are seen, whereas cognitive manifestation is rare in DM2. In contrast to DM1, a severe congenital form of DM2 has not been reported to date.

3.3 Genetics

3.3.1 Repeat Expansion in DM1 and DM2

Short DNA tandem repeats are observed ubiquitously across the human genome [8]. A small fraction of these repetitive elements are prone to become mutable and highly expanded. Such genomic expansions of simple tandem repeats cause various neurogenetic disorders, including DM. DM1 is caused by an expansion of CTG repeats in the 3' untranslated region (UTR) of the *DMPK* gene [2] (Fig. 3.2). The length of CTG repeats is between 50 and several thousand in DM1, whereas the normal repeat number is between 5 and 37. In general, the length of expanded CTG repeats in blood cells correlates with the age of onset in DM1 [9]. DM2 results from a similar expansion of CCTG repeats in the first intron of the *CNBP/ZNF9* gene [3] (Fig. 3.2). The expanded CCTG repeat in DM2 is much longer than that in DM1, ranging from 75 to over 11,000. Importantly, unlike that seen in DM1, the size of expanded CCTG repeats does not correlate with the age of onset in DM2.

3.3.2 Repeat Instability

The expanded CTG and CCTG repeats in DM are not stable [10]. These mutations exhibit an exceptional degree of genetic instability in germline and somatic cells. For instance, the size of the repeat is prone to change during transmission from one generation to the next. In fact, intergenerational increments in the order of hundreds of repeats are often seen in DM1 (intergenerational instability). These mutations are

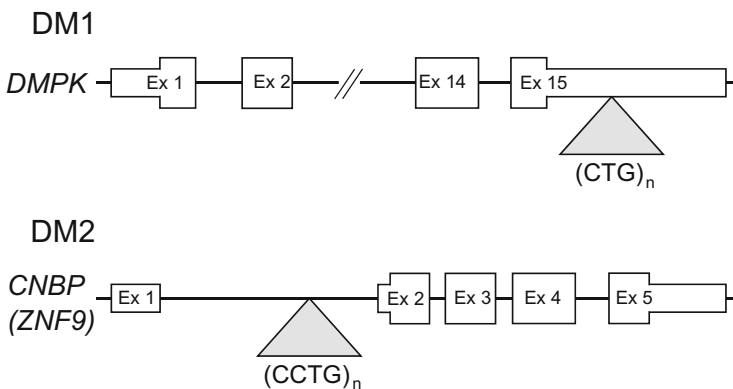


Fig. 3.2 Location of unstable CTG (DM1) or CCTG (DM2) repeats within their respective genes

also unstable in somatic cells, leading to an age-dependent growth of repeat expansion during the life of an individual (somatic instability).

3.3.2.1 Intergenerational Instability

The expanded CTG repeats in DM1 often increase in size when transmitted from parent to offspring, resulting in earlier onset and more severe clinical symptoms in subsequent generations, a phenomenon called “anticipation.” A maternal expansion bias is evident in DM1 and cases with CDM are almost exclusively maternal in origin. There are a few cases of repeat contraction through paternal transmission [11]. In contrast to DM1, anticipation is less evident, both clinically and genetically, in DM2.

3.3.2.2 Somatic Instability

The expansion process continues throughout life in somatic cells, at rates that are variable between tissues in DM. This can lead to over tenfold variations of expansion length in different tissues of a DM individual [12]. Progenitor alleles that are estimated in leukocytes in adult-onset DM1 are usually between 100 and 800 CTG repeat, whereas adult biopsy and autopsy samples show typically 2,000–6,000 CTG repeats in the muscle or heart. In DM2, the CCTG expansion length is also considerably longer in the muscle than in leukocytes from the same individual [13]. Since the heterogeneity in fetal tissues is much less extensive, somatic instability causing these highly expanded alleles occurs mainly during postnatal life. The functional significance of somatic instability remains unclear; however, the progression of DM may be depending on the growth of the expanded repeat over time.

3.3.2.3 Mechanism of Repeat Instability

Instability of the expanded repeats is associated with DNA replication, repair, or transcription, which instigate separation of double-stranded DNA, thus promoting the formation of extrahelical slipped strand structures that are substrates for error-prone repair (Fig. 3.3a). Recent studies have shown that the effects of transcription on repeat stability are particularly pertinent for DM1 [14]. The predominance of transcription-induced instability in DM is reasonable as the organs most affected, such as the skeletal muscle, heart, and brain, are those with low rates of cell proliferation but the highest levels of *DMPK* expression. Recent studies also indicate a role for RNA repeats in transcription-induced repeat instability. RNAs comprised of expanded CUG or CCUG repeats bind to the template strand of DNA, then form RNA:DNA hybrids (R-loops) (Fig. 3.3b). The presence of R-loops

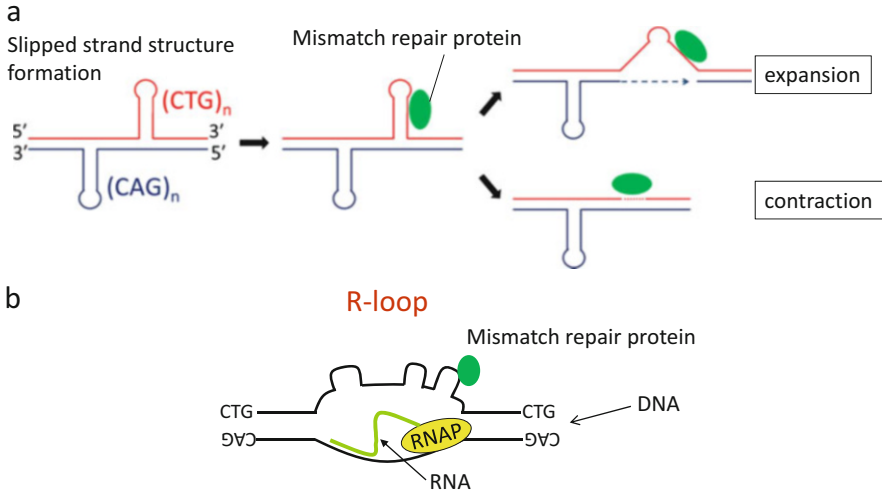


Fig. 3.3 (a) Mechanism of repeat instability. Extrahelical slipped strand structures form in (CTG)_n•(CAG)_n repeats, resulting in error-prone processing by mismatch repair proteins. (b) R-loop formation at the *DMPK* locus. In DM1, expanded CUG RNA binds to the template strand of DNA, then forms RNA:DNA hybrids (R-loops). The presence of R-loops instigates the formation of slipped strand structures on the non-template strand, thereby leading to further repeat instability. RNAP: RNA polymerase

triggers the formation of extrahelical looped-out structures on the non-template strand and thereby stimulates error-prone repair, leading to further expansion or contraction [15].

3.3.3 Genotype-Phenotype Correlation

Although the size of expanded CTG repeats in leukocytes correlates with the age of onset in DM1, there was no correlation between muscle weakness and CTG expansion size in either leukocytes or muscle cells [16]. This suggests that some DM1 patients may tolerate longer expansions better than others and raises the possibility that DM1 severity is modulated by additional factors, such as modifier genes, sequence interruptions in the CTG repeat tract, or epigenetic changes at the *DMPK* locus. The CTG expansion length in the muscle also did not correlate with that in leukocyte from the same subjects [16, 17]. However, the difference in repeat size between leukocytes and muscle is correlated with age, suggesting an age-dependent process of somatic expansion that is more pronounced in the muscle than in leukocytes.

3.4 Pathomechanism

3.4.1 *Unstable Repeat Expansion*

More than 20 neurogenetic disorders are caused by unstable genomic expansions of simple tandem repeats [8]. Such unstable repeat expansion disorders fall in two categories according to the position of the repeat element within the mutant gene. In most cases the repeat tract is located in protein-coding regions, the motif is CAG, and the orientation in the reading frame produces expanded polyglutamine proteins, resulting in neurotoxicity (e.g., Huntington disease, spinocerebellar ataxia types 1, 2, 3, and 6). The second major group of unstable repeat expansion disorders results from repeats in non-protein-coding regions of genes, such as expanded CGG repeats in the 5' UTR of *FMRI* in fragile X syndrome (FXS) [18], GAA repeats in the first intron of *FXN* in Friedreich's ataxia (FRDA) [19], and CTG/CCTG repeats in DM. In cases with FXS and FRDA, the critical effect of the expanded repeats is transcriptional silencing of *FMRI* and *FXN*, respectively. However, there is no proven effect on transcription of *DMPK* or *CNBP/ZNF9* in DM, indicating that the main pathogenic effect is a deleterious gain of function by the mutant mRNA.

3.4.2 *RNA Dominance*

Although the mutant mRNA is fully processed in DM1, repeat expansion blocks the nuclear export of mRNA with the expanded repeats of CUG (CUG^{exp}) [20]. Thus, the mRNA containing expanded repeats is retained in the nucleus and accumulates in discrete foci. Since DM1 is an autosomal dominant disease, nuclear retention of the mutant mRNA could theoretically lead to a 50 % reduction in DMPK protein. However, several lines of evidence strongly indicate that symptoms of DM1 result not from haploinsufficiency but from the toxic gain of function by RNA transcripts containing the CUG^{exp}. First, *DMPK* knockout mice, in which DMPK protein can be completely eliminated, show only minor symptoms in the skeletal muscle [21, 22]. Second, muscular features of DM1 can be reproduced by the expression of CUG^{exp} RNA at the 3' UTR of human skeletal actin transgene in a mouse model [23]. Finally, in DM2, the highly expanded CCUG repeat in *CNBP/ZNF9* is fully transcribed, but the intron containing the CCUG expansion is retained and accumulated in nuclear foci similar to that seen in DM1 [3, 24].

3.4.3 *RNA-Binding Proteins: MBNL Family and CELF1*

The mutant RNA retained in the nucleus affects at least two RNA-binding protein families: the MBNL and CELF. MBNL1, a member of the MBNL family protein,

has a strong affinity for CUG^{exp} and CCUG^{exp} [25]. When CUG^{exp} or CCUG^{exp} RNA accumulates in the nucleus, MBNL1 is sequestered in nuclear RNA foci and depleted from the nucleoplasm [26, 27] (Fig. 3.4). Since MBNL1 is a regulator of alternative splicing [28], MBNL1 sequestration in foci leads to the misregulation of alternative splicing of its target exons. One clear example of the functional consequence in DM is the splicing misregulation of *CLCN1* [29, 30]. This gene encodes a chloride ion channel that stabilizes the transmembrane potential in skeletal muscle. When alternative splicing of *CLCN1* is misregulated in response to expression of CUG^{exp} RNA, the channel activity is lost, causing electrical instability of the membrane, which triggers repetitive action potentials and delay of relaxation (myotonia). The loss of MBNL1 has additional effects on gene expression, including transcriptional alterations, mRNA decay, transport of mRNA, and micro-RNA biogenesis [31–33]. *Mbnl1* knockout mice exhibit myotonia and splicing alteration of *Clcn1*, supporting the evidence for MBNL1 dysfunction in DM1 [34]. Two other members of the MBNL family, MBNL2 and MBNL3, may also be involved in DM [34, 35]. Similar to MBNL1, MBNL2 is expressed in the skeletal muscle, heart, and brain, whereas MBNL3 is expressed mainly in the placenta. MBNL2 and MBNL3 are closely related to MBNL1 and can also be sequestered in nuclear foci. These suggest a role for the combinatorial deficiency of all three MBNL family members in DM, depending on the expression of CUG^{exp} or CCUG^{exp} RNA relative to the distinct levels of MBNL proteins in different tissues. Indeed, a recent report showed compound loss of MBNL1 and MBNL2 functions exaggerated cardiac and skeletal muscle symptoms in a mouse model [36].

Expression of CUG^{exp} also causes perturbations in cell signaling via several kinases, including protein kinase C (PKC), Akt kinase, cyclin-dependent kinase 4 (CDK4), and glycogen synthase kinase 3 beta (GSK3B) [37–39]. While the mechanisms of activation of these pathways have not yet been determined, upregulation of PKC enhances the phosphorylation of CELF1, a member of CELF RNA-binding protein family. The phosphorylation of CELF1 extends its half-life, resulting in the upregulation of its steady-state level [37] (Fig. 3.4). Since CELF1 is a multifunctional RNA-binding protein involved in the regulation of alternative splicing and translation, upregulation of CELF1 protein in DM1 results in misregulated alternative splicing of several pre-mRNAs, as well as altered translation or decay of mRNAs that are bound by CELF1 [40–43]. Transgenic mice overexpressing *Celf1* show splicing misregulation and cardiac conduction defect similar to DM1 [44]. As CELF1 plays an important role in developmental splicing switch in the heart, increased CELF1 expression may underlie the cardiac pathology observed in DM1. However, CELF1 upregulation has not been confirmed in DM2 [27, 45].

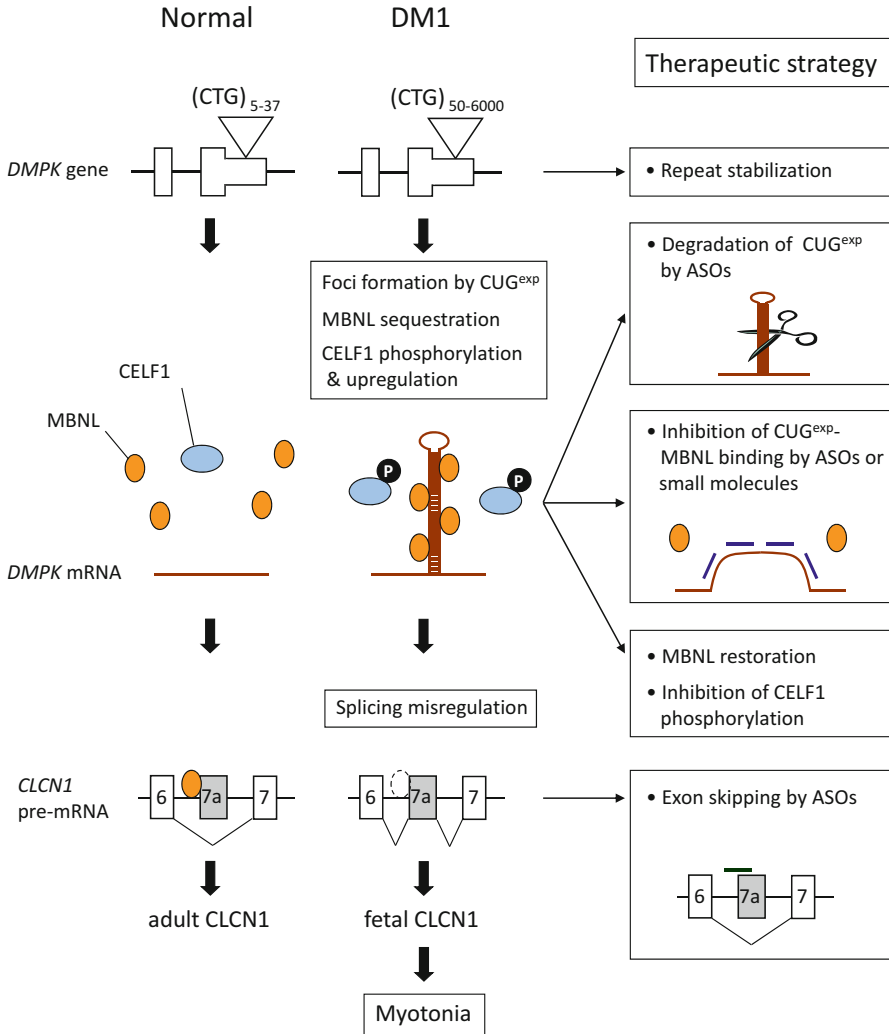


Fig. 3.4 Schematic illustration of the RNA-mediated disease mechanism (*left*) and possible therapeutic strategies (*right*) in DM1. Expanded CUG repeats in the mutant *DMPK* mRNA form hairpin structures and nuclear foci, sequester MBNL1 in the nucleus, and phosphorylate and stabilize CELF1. Loss of functional MBNL1 and upregulation of CELF1 cause misregulation of alternative splicing. Mis-splicing of *CLCN1* exon 7a induces a frameshift and premature termination codon in exon 7, resulting in loss of functional CLCN1 protein on the sarcolemma and myotonia in DM1. Therapeutic strategies are currently being developed for DM, including (1) stabilization of expanded repeats, (2) degradation of the toxic RNA by ASOs, (3) neutralization of CUG^{exp} toxicity by preventing MBNL sequestration with ASOs or small molecules, (4) modulation of MBNL or CELF1, and (5) induction of exon skipping of individual targets (Figure modified from Ref. [82])

3.4.4 Spliceopathy in DM

RNA splicing is the mechanism by which intervening introns are removed, while functional exons are ligated together to form a mature mRNA transcript [46]. There are two types of RNA splicing: constitutive and alternative splicing. Alternative splicing serves to generate a wide variety of unique mRNA transcripts by selecting and pairing discrete exons, resulting in distinct but similar proteins from the same gene (Fig. 3.5).

Although many human genetic disorders have been identified as the consequences of mutations on RNA splicing, in almost all cases, these are cis-acting effects that affect the splicing of a single pre-mRNA. In contrast, DM is caused by a trans-effect of alternative splicing factors on many RNAs and now is recognized as the first example of a human genetic disease resulting from “spliceopathy” [4]. The spliceopathy in DM shares common features in splicing misregulation. First, it does not involve constitutive exons, but selectively affects a group of exons that are subject to alternative splicing. Second, it usually affects alternative exons that normally undergo a developmental splicing switch; therefore, the fetal or neonatal splicing isoforms are expressed in mature muscle fibers in DM1. Third, most target exons in the spliceopathy in DM are regulated by MBNL1, CELF1, or both.

More than 70 mis-splicing events have been reported in DM. Table 3.1 lists the transcripts known to be affected by spliceopathy in DM. In case of *CLCN1*, the

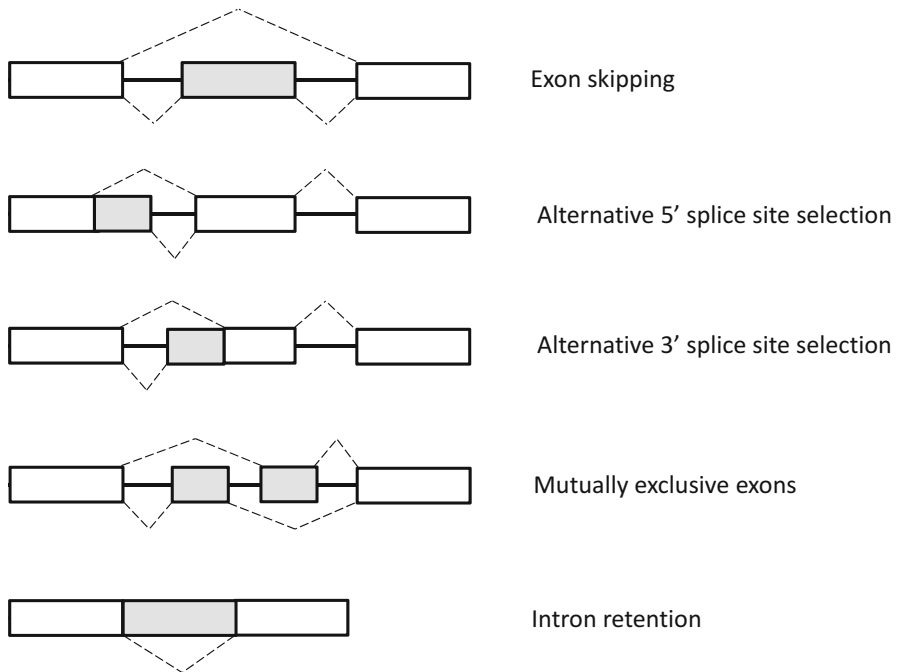


Fig. 3.5 Patterns of alternative splicing. Constitutive exons present in all final mRNAs are indicated by *white* boxes. Alternative RNA segments that may or may not be included in the final mRNA are indicated by *gray* boxes

Table 3.1 Mis-splicing events described in DM

Gene symbol	Function of gene product	Gene symbol	Function of gene product
Skeletal muscle		Heart	
Cl^- ion transport		Cytoskeletal component	
<i>CLCN1</i>	Chloride channel	<i>TNNT2</i>	Troponin T
		<i>DMD</i>	Dystrophin
Ca^{2+} homeostasis		<i>DTNA</i>	Dystrobrevin
<i>RYR1</i>	Ryanodine receptor	<i>TTN</i>	Titin
<i>ATP2A1</i>	Calcium ATPase	<i>LDB3</i>	Z-disk
<i>ATP2A2</i>	Calcium ATPase	<i>PDLIM3</i>	Z-disk
<i>BIN1</i>	T-Tubule formation	<i>CAPZB</i>	Assembly of actin filament
<i>CACNA1S</i>	Calcium channel		
		<i>Ion channel</i>	
<i>Cytoskeletal component</i>		<i>KCNAB1</i>	Potassium channel
<i>DMD</i>	Dystrophin	<i>SCN5A</i>	Sodium channel
<i>DTNA</i>	Dystrobrevin		
<i>TTN</i>	Titin	<i>Others</i>	
<i>LDB3</i>	Z-disk	<i>MTMR1</i>	Phosphatase activity
<i>CAPZB</i>	Assembly of actin filament		
<i>TNNT3</i>	Troponin T		
<i>PDLIM3</i>	Z-disk		
<i>PDLIM7</i>	Z-disk		
<i>FHOD1</i>	Actin organization		
<i>NRAP</i>	Myofibril assembly		
<i>ABLIM2</i>	Z-disk	<i>Brain</i>	
<i>MYBPC1</i>	Myosin-binding protein C	<i>APP</i>	Amyloid beta precursor
<i>MYOM1</i>	Z-disk	<i>GRIN1</i>	NMDA receptor
		<i>MAPT</i>	Tau
<i>Signaling</i>		<i>CAMK2D</i>	Signaling
<i>INSR</i>	Insulin receptor	<i>SORBS1</i>	Signaling
<i>SOS1</i>	Receptor signaling	<i>DCLK1</i>	Microtubule polymerization
<i>ALPK3</i>	Myogenesis	<i>MPRIP</i>	Rho interacting
<i>CAMK2B</i>	Signaling	<i>TANC2</i>	Development
<i>MAP4K4</i>	Signaling	<i>KCNMA1</i>	Potassium channel
		<i>CSNK1D</i>	Casein kinase
<i>Transcription</i>		<i>CACNA1D</i>	Calcium channel
<i>MBNL1</i>	Alternative splicing	<i>LIMCH1</i>	Actin binding
<i>MBNL2</i>	Alternative splicing	<i>ADD1</i>	Cytoskeletal protein
<i>NFIX</i>	Transcription factor	<i>PPP1R12A</i>	Myosin phosphatase

(continued)

Table 3.1 (continued)

Gene symbol	Function of gene product	Gene symbol	Function of gene product
Skeletal muscle		Heart	
<i>MEF2C</i>	Transcription factor	<i>CLASP2</i>	CLIP binding
<i>NCOR2</i>	Transcriptional silencing	<i>RYR2</i>	Ryanodine receptor
<i>FXR1</i>	mRNA trafficking		
<i>Others</i>			
<i>GFPT1</i>	Protein glycosylation		
<i>IMPDH2</i>	Nucleotide biosynthesis		
<i>MTMR1</i>	Phosphatase activity		
<i>CAPN3</i>	Intracellular protease		
<i>PHKA1</i>	Glycogenesis		
<i>PHKA2</i>	Glycogenesis		
<i>KIF13A</i>	Endosome positioning		
<i>VPS39</i>	Vesicle trafficking		
<i>ARFGAP2</i>	Vesicle trafficking		
<i>COPZ2</i>	Vesicle trafficking		
<i>TBC1D15</i>	Intracellular trafficking		
<i>ANK2</i>	Membrane targeting		
<i>OPA1</i>	Mitochondrial dynamics		
<i>UBE2D3</i>	Ubiquitination		
<i>USP25</i>	Deubiquitination		
<i>TXNL4A</i>	PQBP1 binding protein		
<i>VEGFA</i>	Angiogenesis		
<i>MAPT</i>	Tau		
<i>MLF1</i>	Oncoprotein		

exon 7a is negatively regulated by MBNL1 [34]. When MBNL1 is depleted in DM muscle, the exon 7a is retained in the mRNA. The inclusion of exon 7a induces a premature termination codon, which causes loss of mature CLCN1 protein in muscle membrane in DM, resulting in myotonia [29]. Misregulated splicing of insulin receptor (*INSR*) is also reported in DM muscle [41]. Alternative splicing of exon 11 is an important factor dictating the function of this receptor. The receptor lacking the exon 11 (non-muscle form) has a higher affinity for insulin than the receptor including the exon (muscle form). Because the inclusion of exon 11 is negatively regulated by CELF1, the non-muscle form lacking exon 11 is increased in DM muscle, leading to insulin resistance. However, whereas numerous mis-splicing events have been identified in DM, the underlying cause of progressive muscle wasting, the main disabling symptom in DM, has not been well defined. One possible candidate is mis-splicing of a group of genes regulating Ca²⁺ homeostasis,

such as ryanodine receptor (*RYR1*), sarcoplasmic/endoplasmic reticulum calcium ATPases (*SERCAs*), voltage-dependent Ca^{2+} channel (*CACNA1S*), and T-tubule membrane scaffolding protein (*BINI1*) [47–49]. Because Ca^{2+} concentration is increased in DM1 cells and impaired Ca^{2+} homeostasis triggers muscle damage, misregulated splicing of these transcripts may cause muscle wasting in DM [50]. Another candidate is splicing misregulation of genes encoding cytoskeletal proteins, such as dystrophin (*DMD*), dystrobrevin (*DTNA*), titin (*TTN*), and ZASP (*LDB3*), as mutations or deletions in these genes causes other types of muscular dystrophies and myopathies [27, 51, 52].

The spliceopathy in DM also involves the heart and brain. Although the cause of cardiac symptoms in DM is not well elucidated, mis-splicing of cardiac cytoskeletal proteins (cardiac troponin T, *DMD*, *DTNA*, *TTN*, and *LDB3*) may contribute to cardiomyopathy [51–54]. In addition, conduction block and arrhythmia in DM may be caused by mis-splicing of cardiac ion channels, such as K^+ channel beta subunit (*KCNAB1*) and voltage-gated Na^+ channel (*SCN5A*) [54] (Charlet-Berguerand & Takahashi, manuscript in preparation). In DM1 brain, several splicing misregulations have been reported, including Tau (*MAPT*), *APP*, and *CAMK2D* [26, 55]. However, neither the functional consequences of these mis-splicing events nor the cause of cognitive impairment in DM1 has been determined.

3.4.5 Other Possible Mechanisms

Besides MBNL and CELF1, other RNA-binding proteins, such as hnRNP H and staufen, are implicated in DM pathogenesis [56, 57]. Dysregulation of micro-RNAs and MEF2 transcription factor proteins are also suggested to contribute to cardiac symptoms in DM [33, 58]. Furthermore, repeat-associated non-ATG translation of the *DMPK* antisense transcript was shown to give rise to polyglutamine aggregates [59]. Future studies are needed to elucidate the role of each mechanism in DM pathogenesis.

3.5 Therapeutic Strategies

Although no curative treatment exists in DM at present, recent advances in research and pharmacological approaches provide clues for potential future therapeutic intervention. Along with a better understanding of disease mechanisms such as the spliceopathy because of RNA toxicity, several experimental approaches have been developed and applied to translational research in DM (Fig. 3.4).

3.5.1 *Splicing Correction*

Since splicing misregulation is the molecular basis of DM, correction of individual mis-splicing events is the most direct way to alleviate the symptoms. Recent advances in technology of antisense oligonucleotides (ASOs) has enabled this strategy. ASOs are short, synthetic, modified nucleic acids that bind complementary RNA and regulate its function [60]. ASOs modulate splicing events by hiding specific sites essential for exon inclusion from the splicing machinery. ASOs blocking target splice sites can skip the target exon(s) and restore normal splicing. This approach has been applied as a treatment for Duchenne muscular dystrophy (DMD). The exon skipping achieved by ASOs restores dystrophin expression in DMD patients and is currently evaluated by clinical trials [61]. In a mouse model of DM1, a morpholino ASO targeting the 3' splice site of *Clcn1* exon 7a reversed the defect of alternative splicing [62]. The morpholino ASOs repressed the inclusion of exon 7a, restored the normal *Clcn1* mRNA, increased the expression of CLCN1 protein on sarcolemma, stabilized the membrane potential and, thereby, alleviated myotonia. These findings suggest that ASO-induced exon skipping is a powerful tool for correcting mis-splicing in DM. However, there are several hurdles to overcome in the application of this approach in DM patients: (1) alternative splicing of *CLCN1* is more complicated in humans than in mice and (2) mis-splicing events responsible for other symptoms, especially muscle wasting, are not known.

3.5.2 *Modulation of Alternative Splicing Factors*

In DM, MBNL1 sequestration leads to multiple mis-splicing events, such as *CLCN1* and *ATP2A1* [34]. One therapeutic approach is the restoration of MBNL1 protein in the nucleoplasm. Overexpression of MBNL1 by adeno-associated viral gene delivery in skeletal muscle of mice expressing CUG^{exp} reversed MBNL1-dependent mis-splicing of exons and rescued myotonia [63]. However, the MBNL1 overexpression in a DM1 mouse model did not improve muscle pathology. In addition, *Mbnl1* knockout mice neither reproduce the developmental features of CDM nor display the severe muscle wastings that occur in adult-onset DM1 [34]. Thus, as MBNL1 sequestration does not provide a unifying explanation for the disease process, simultaneous modulation of other splicing factors, such as MBNL2 and CELF1, may be required for successful treatment of DM.

In addition to MBNL1 sequestration, increased steady-state level of CELF1 protein is another important molecular event involved in DM1 pathogenesis [64]. Overexpression of CELF1 in the mouse heart and skeletal muscle causes DM1-associated splicing changes and results in disease phenotypes [44, 65, 66]. Activation of the PKC signaling pathway is suggested to leads to the hyperphosphorylation and stabilization of CELF1 [37]. A recent report showed that PKC inhibitors reduced phosphorylation of CELF1, decreased the steady-state levels,

reversed misregulation of splicing events regulated by CELF1, and ameliorated the cardiac conduction defects and contraction abnormalities in a heart-specific DM1 mouse model [67]. Although this approach may be promising for the treatment of cardiac symptoms in DM, there remain important unanswered questions: (1) why is CELF1 not consistently increased in DM2 despite cardiac involvement similar to DM1, and (2) how do CUG^{exp} activate the PKC signaling pathway?

3.5.3 Targeting Toxic RNA

In DM, the mutant RNA exerts a pivotal role in trans-dominant effects on different transcripts and spliceopathy. Thus, interventional strategies against toxic RNA are ideal to accomplish a generalized correction of DM symptoms. Several experimental therapeutic approaches have been attempted to degrade the toxic RNA by small interfering or short hairpin RNA delivered with a retroviral vector and hammerhead ribozyme [68–70]. Although these RNA-based approaches reduced the mutant RNA in DM1 myoblast cells, normal *DMPK* mRNA was simultaneously decreased, raising concerns regarding the loss of endogenous DMPK function. In addition, unmodified nucleic acids are susceptible to cleavage by endogenous nucleases and are rapidly degraded in vivo. From this view point, chemically modified ASOs with high binding affinity and extreme nuclease resistance emerge as a suitable tool for the modulation of toxic RNAs in DM. Depending on the chemistry and sequence, ASO binding can modulate the target RNA metabolism by two distinct mechanisms: neutralization and degradation. Neutralizing ASOs bind to the target RNA and sterically hinder RNA-protein interactions. Therefore, CAG-repeat ASOs can be expected to bind CUG^{exp}, compete with MBNL proteins, and prevent MBNL sequestration in DM1. In fact, recent reports showed that CAG-repeat ASOs with different chemistry (2-*O*-methyl, morpholino, locked nucleic acid) mitigated the RNA toxicity in DM1 model mice [15, 71, 72]. Most strikingly, CAG-morpholino ASOs dispersed nuclear foci of CUG^{exp}, released MBNL1 from foci, corrected mis-splicing events, and eliminated myotonia [72]. One caveat with this approach is the delivery of the ASOs by direct injection into the muscle, followed by electroporation, an approach that is not practical for human patients. However, systemic administration of CAG-ASOs with peptide-linked morpholino (PPMO) modification was reported to improve myotonia in a DM1 mouse model, providing the proof-of-concept for clinical applications given the versatility of CAG-ASOs [73] (see also Sect. 3.5.4). In contrast to these neutralizing ASOs, target-degrading ASOs cleave target RNA by recruiting RNase H to the oligonucleotide-RNA heteroduplex. This degradation capability arises from a characteristic structure called “gapmer” that has chemically modified residues on either side of a stretch of unmodified residues referred to as the gap. The modified wings enhance the binding and stability of the ASOs, and the gap allows for the induction of RNase H activity. Because of the exceptional capability of target degradation, gapmer ASOs are considered as promising for DM treatment and have already been tested in DM1 model mice [15, 74, 75].

In one study, systemic administration of the gapmer ASO with 2-*O*-methyl modification decreased toxic RNA, reduced foci formation, corrected splicing defects, and eliminated myotonia in DM1 mouse model [74]. More importantly, these effects were sustained for up to one year after the discontinuation of treatment. This approach achieved the best therapeutic effects among all strategies in mice so far and led to the development of gapmer ASOs against human *DMPK*. As therapeutic effects were confirmed in a mouse model [74], the gapmer ASOs (ISIS-DMPKRx) will hopefully be applied to a clinical trial soon.

Small molecules are also appropriate tools for targeting mutant RNA. Recent studies have identified numerous small molecules that bind specifically the CUG or CCUG repeats and competitively release the sequestered MBNL1. Several of these molecules were shown to induce the dispersal of RNA foci, redistribution of MBNL1 to the nucleoplasm, and partial reversal of mis-splicing events in mice expressing CUG^{exp} [76–78]. However, toxicity of these compounds in human has not been determined, and none of the candidates achieved a full rescue of mis-splicing of *Cln1* and myotonia in mice. Pentamidine and related compounds have been recently suggested to inhibit the transcription of CUG^{exp} by binding to the CTG repeat DNA [79, 80]. Indeed, heptamidine, a methylene linker analogue of pentamidine, reversed splicing defects and improved myotonia in the mouse model.

3.5.4 Stabilization of Expanded Repeats

Another striking feature of DM is the marked instability of the expanded repeats. The progression of DM1 may depend on the growth of the expanded repeat throughout life. The ongoing repeat expansion also raises a concern that an initial beneficial effect of therapeutic ASOs on the toxic RNA may be lost because of ongoing expansion and increased production of CUG^{exp} RNA. Therefore, stabilization of the repeat is also a potential approach to postpone the onset, slow the progression, or to sustain the effect of therapeutic ASOs. Several compounds that stabilize the repeats in cultured cells have been identified [81]. However, these compounds are DNA intercalators or drugs that affect DNA methylation or replication, evoking concerns about long-term safety. In DM1, the instability of expanded repeats is enhanced by transcription across the repeat and R-loop formation [14, 15]. Because the R-loop is formed with CUG RNA and CAG DNA on the template strand, CAG-ASOs can theoretically prevent its formation. Indeed, a recent report showed that CAG-ASOs suppressed the repeat instability in both a cellular model and a mouse model by reducing the formation of R-loops at the *DMPK* locus [15]. Thus, intervention with CAG-repeat ASOs has the dual benefit of preventing the RNA toxicity and stabilizing the repeat.

3.6 Concluding Remarks

Since the CTG expansion was discovered in DM1, the understanding of the molecular pathomechanisms in DM has been enormously advanced. Promising experimental therapeutic strategies targeting toxic RNA continue to emerge. A recent study showed that several mis-splicing events can be a good biomarker for disease severity and therapeutic response in DM [16]. National and international DM patient registries have already been established in several countries for a clinical trial. Indeed, gapmer-ASOs targeting mutant *DMPK* mRNA for treatment of DM1 is currently being planned for a first-in-human clinical trial. Combination therapy with small molecules is also an attractive option. These developments are opening a new, fascinating, and promising era for the treatment of DM.

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