

# Pathogenic mechanisms of myotonic dystrophy

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## Abstract

DM (myotonic dystrophy) is a dominantly inherited genetic disorder that is the most common cause of muscular dystrophy in adults affecting 1 in 8500 individuals worldwide. Different microsatellite expansions in two loci cause different forms of the disease that share similar features: DM1 (DM type 1) is caused by a tri- (CTG) nucleotide expansion within the DMPK (dystrophin myotonia protein kinase) 3'-untranslated region and DM2 (DM type 2) is caused by a tetra- (CCTG) nucleotide expansion within intron 1 of the *ZNF9* (zinc finger 9) gene. The pathogenic mechanism of this disease involves the RNA transcribed from the expanded allele containing long tracts of (CUG)<sub>n</sub> or (CCUG)<sub>n</sub>. The RNA results in a toxic effect through two RNA-binding proteins: MBNL1 (muscleblind-like 1) and CUGBP1 (CUG-binding protein 1). In DM1, MBNL1 is sequestered on CUG repeat-containing RNA resulting in its loss-of-function, while CUGBP1 is up-regulated through a signalling pathway. The downstream effects include disrupted regulation of alternative splicing, mRNA translation and mRNA stability, which contribute to the multiple features of DM1. This review will focus on the RNA gain-of-function disease mechanism, the important roles of MBNL1 and CUGBP1 in DM1, and the relevance to other RNA dominant disorders.

## Introduction

DM (myotonic dystrophy) is a multisystemic disorder and the second most common form of muscular dystrophy. Major features of the disease include myotonia (hyperexcitability of skeletal muscle), muscle wasting, insulin resistance, cardiac conduction defects, cataracts, cognitive dysfunction and mental retardation in the most severe congenital form of the disease. DM is among the class of diseases called microsatellite expansion disorders in which a tandem nucleotide repeat within a specific gene, typically containing a variable number of repeats within the population, expands to a pathogenic range. More than 20 microsatellite expansion disorders have been described [1]. In addition to DM, this class of diseases includes Huntington's disease, SCA (spinocerebellar ataxia) and fragile X syndrome. There are two types of DM caused by microsatellite expansions in different genes. DM1 (DM type 1) is the more common of two forms in the United States. Our understanding of the disease has increased enormously over the past decade. This review discusses the important findings regarding the pathogenic mechanisms of DM1.

## Evidence of RNA gain-of-function

In 1992, the DM1 mutation was found to be expanded (up to >4000) CTG repeats located within the 3'-UTR (3'-untranslated region) of the *DMPK* (dystrophin myotonia

protein kinase) gene in exon 15 [2]. Unaffected individuals contain 5–38 CTG repeats, while symptoms occur in individuals with as few as 50 repeats. Larger repeat size correlates with increased severity of symptoms and decreased age of onset [3]. Several hypotheses have been proposed to explain the pathogenesis of this multisystemic disorder. One is that the repeats inhibit *DMPK* mRNA or protein production, resulting in *DMPK* haploinsufficiency. This was supported by studies demonstrating decreased expression of *DMPK* mRNA and protein in DM1 muscle [4]. Surprisingly, *Dmpk*-knockout mice displayed only mild myopathy [5] and cardiac conduction defects in older animals [6]. More importantly, the mice did not exhibit myotonia, one of the characteristic symptoms of DM1. Therefore the multisystemic features of DM1 cannot be explained solely by *DMPK* haploinsufficiency. To date, no *DMPK* loss-of-function mutation has been identified in DM1 patients.

Although *DMPK* haploinsufficiency is not the predominant cause of the disease, the CTG repeats might influence expression of adjacent genes. A nuclease resistant region indicating condensed chromatin structure was found downstream of the CTG repeat expansion [7]. A homoeodomain-encoding gene named *SIX5* is located in this region and its mRNA level is indeed decreased in DM1 patients [8]. However, *Six5*-knockout mice develop only cataracts and no muscle pathology was observed [9]. Therefore decreased *SIX5* expression is not the primary cause of DM1.

The RNA gain-of-function hypothesis proposes that the mutant RNA transcribed from the expanded allele is sufficient to induce symptoms of the disease. This was suggested by several observations: (i) loss of function of *DMPK* or surrounding genes did not reproduce major features of DM1 [5,9], (ii) the expanded CTG repeats are transcribed into

**Key words:** CUG repeat, CUG-binding protein 1 (CUGBP1), dystrophin myotonia protein kinase (DMPK), muscleblind-like 1 (MBNL1), myotonic dystrophy type 1 (DM1), RNA gain-of-function.

**Abbreviations used:** AAV, adeno-associated virus; ARE, AU-rich element; CIC-1, chloride channel 1; cTNT, cardiac troponin T; CUGBP1, CUG-binding protein 1; DM, myotonic dystrophy; DM1, DM type 1; DMPK, dystrophin myotonia protein kinase; FMR1, fragile X mental retardation 1; FXTAS, fragile X associated tremor/ataxia syndrome; IR, insulin receptor; MBNL1, muscleblind-like 1; MEF2A, myocyte enhancer factor 2A; PKC, protein kinase C; SCA, spinocerebellar ataxia; TNF, tumour necrosis factor; UTR, untranslated region.

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CUG repeats that accumulate in discrete nuclear foci [10], (iii) expression of only the *DMPK* 3'-UTR with 200 CTG repeats is sufficient to inhibit myogenesis [11]. Direct experimental support of the RNA gain-of-function hypothesis was provided by a mouse model (*HSA<sup>LR</sup>*) expressing 250 CTG repeats in the 3'-UTR of the human skeletal  $\alpha$ -actin gene. These mice develop myotonia and exhibit muscle histology similar to that of DM1, including increased central nuclei and ring fibres [12]. This demonstrates that CUG repeats alone, regardless of the gene context, are sufficient to induce pathogenic features of DM1. Additional mouse models for DM1 further support the RNA gain-of-function model. Seznec et al. [13] generated transgenic mice that carry a large genomic fragment containing either the normal (20 CTGs, DM20) or mutant (>300 CTGs, DM300) allele of *DMPK* as well as two flanking genes. DM300 mice developed DM1-like phenotype, while DM20 mice were normal, suggesting that the length of the repeats is responsible for the phenotype [13]. Two inducible mouse models expressing 960 CUG repeats in the *DMPK* 3'-UTR specifically in heart or muscle also recapitulate DM1 symptoms [14,15].

In 1998, a second type of DM not caused by the DM1 mutation was identified and termed DM2 (DM type 2) [16]. DM2 patients have symptoms similar to DM1, with the exception that no congenital form of DM2 has been reported. DM2 results from CCTG repeats within intron 1 of the *ZNF9* (zinc finger 9) gene [17]. The fact that two repeat sequences located in entirely different genes can cause such similar disease features implies a common pathogenic mechanism by RNA gain-of-function. In summary, it is now clear that the mutant RNA is the predominant cause of DM1 pathogenesis, although loss of *DMPK* and *SIX5* may also play a minor role.

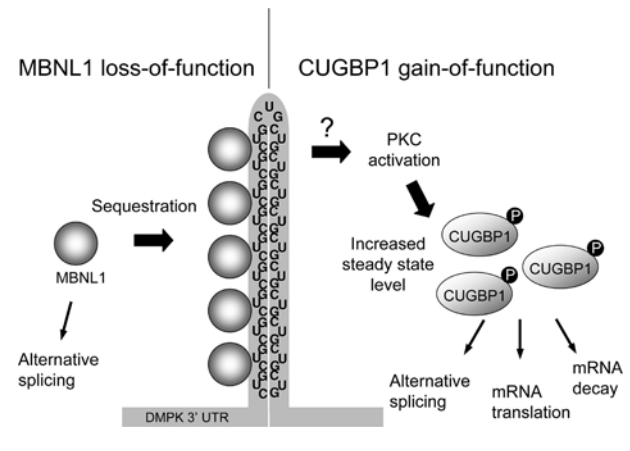
One explanation of how repeat-containing RNA can cause disease symptoms is through interaction with RNA-binding proteins. A CUG expansion of more than 11 repeats tends to fold into a hairpin-like secondary structure in which C-G base pairs are interrupted by U-U mismatches [18] (Figure 1). It was proposed that specific proteins are sequestered on the double-stranded hairpins, resulting in depletion below a functional threshold. Two important proteins were identified by their ability to bind to CUG repeats: MBNL1 (muscleblind-like 1) and CUGBP1 (CUG-binding protein 1), which will be the focus of the following sections.

## MBNL1

MBNL1 was identified in a screen for proteins that specifically bound to long expansions of CUG repeats [19]. The *Drosophila* homologue of MBNL1 is essential for muscle terminal differentiation [20], suggestive of an ideal candidate for the protein sequestration model. MBNL1 co-localizes to CUG-containing nuclear foci in DM1 cells and is depleted from the nucleoplasm [21,22]. To determine the role of MBNL1 in DM1, Kanadia et al. [23] generated knockout mice (*Mbnl1<sup>ΔE3/ΔE3</sup>*) with targeted deletion of *Mbnl1* exon 3 where an RNA-binding motif is located. Eliminating the CUG-binding isoforms of *Mbnl1* mimics

## Figure 1 | Expanded CUG repeats in DM1 result in MBNL1 loss-of-function and CUGBP1 gain-of-function

MBNL1 is sequestered to the double-stranded hairpin structure formed by CUG repeats, depleting it from the nucleoplasm. The CUG repeats stimulate PKC activation through an unknown mechanism, which induces CUGBP1 hyperphosphorylation and stabilization. The downstream effects include disruption of alternative splicing, mRNA translation and mRNA decay.



the molecular environment in DM1, where these isoforms are depleted from the nucleoplasm. These mice develop several characteristic features of DM1, including myotonia and cataracts [23]. In addition, AAV (adeno-associated virus)-mediated overexpression of mouse *Mbnl1* in skeletal muscle of *HSA<sup>LR</sup>* mice is sufficient to reverse the myotonia [24]. These data demonstrate that MBNL1 depletion indeed contributes to DM1 pathogenesis, but how? The answer lies in the role of MBNL1 as a regulator of alternative splicing.

An important molecular feature of DM1 is the mis-regulation of alternative splicing. More than two dozen splicing events are mis-regulated in DM1 [25] and some events correlate directly with DM1 symptoms. For example, the myotonia seen in DM1 is due to the abnormal splicing of the skeletal muscle-specific *CLC-1* (chloride channel 1). Increased inclusion of exons containing premature stop codons result in down-regulation of *CLC-1* mRNA and protein [26,27], which is sufficient to cause myotonia. A direct link between *CLC-1* and myotonia was established by a study in which modified DNA oligonucleotides (morpholinos) were introduced into skeletal muscle of *HSA<sup>LR</sup>* mice to reverse the splicing of *CLC-1* and rescue myotonia [28]. Another abnormal splicing event seen in DM1 is increased skipping of the IR (insulin receptor) exon 11. The spliced isoform lacking exon 11 has lower signalling capacity [29] and increased expression correlates with insulin resistance in DM1 individuals [30]. There is also increased inclusion of cTNT (cardiac troponin T) exon 5 in DM1 [31], which possibly contributes to the cardiac conduction defects.

*Mbnl1<sup>ΔE3/ΔE3</sup>* mice display the same splicing defects observed in DM1, strongly suggesting a role of MBNL1 in alternative splicing regulation [23]. Ho et al. [32] showed that knockdown of MBNL1 in cultured cells results in

disrupted splicing of both cTNT and IR. Furthermore, overexpression of MBNL1 in DM1 myoblasts is sufficient to rescue the IR splicing defect [33]. AAV-mediated delivery of Mbnl1 to HSA<sup>LR</sup> skeletal muscle restored normal CIC-1 splicing and reversed myotonia, further demonstrating direct links between MBNL1, CIC-1 splicing and myotonia [24]. MBNL1 promotes skipping of cTNT exon 5 by binding directly to the upstream intron [32], which blocks recruitment of the essential splicing factor U2AF65 [34]. Intriguingly, the MBNL1-binding site on this intron forms a hairpin structure containing mismatches similar to the expanded CUG repeats [35,36]. This implies that MBNL1 is sequestered to the repeat RNA because the secondary structure resembles its natural binding site. An interesting observation is that all of these mis-spliced events are developmentally regulated and the predominant transcript switches from the adult isoform to the embryonic isoform in DM1. MBNL1 localization switches from cytoplasmic to predominantly nuclear during normal skeletal muscle development [22], hence it is proposed that MBNL1 sequestration reverses a subset of splicing targets to their embryonic isoform, contributing to multiple symptoms of DM1.

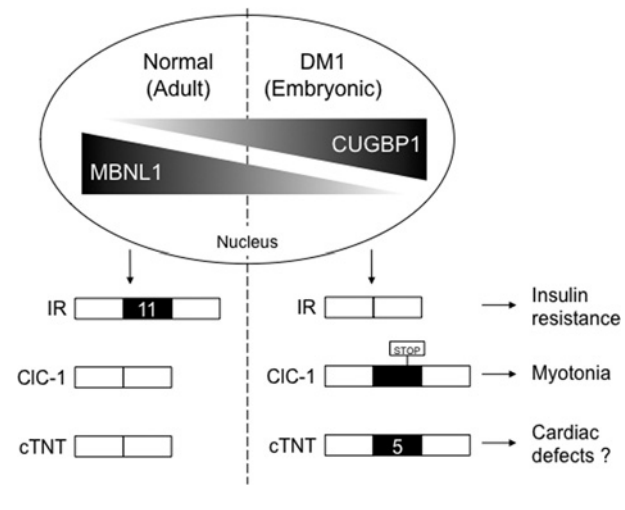
Although the MBNL1 sequestration model (Figure 1) is well established, it is not the only mechanism of DM1 pathogenesis. One example is that Mbnl1<sup>ΔE3/ΔE3</sup> mice do not display muscle wasting, suggesting that some aspects of the disease may not result from loss of MBNL1 function [23]. Also, Ho et al. [37] showed that while MBNL1 can be sequestered to both CUG- and CAG-containing foci with similar affinity, only CUG repeats induced abnormal splicing of cTNT and IR minigenes. Thus MBNL1 sequestration alone may not be sufficient to induce all the splicing changes in this disease.

## CUGBP1

Another protein involved in DM1 is CUGBP1, which was originally identified by its ability to bind short single-stranded CUG repeats [38]. CUGBP1 does not bind to double-stranded CUG repeats or co-localize with RNA nuclear foci [39,40], thus it is not a sequestered factor like MBNL1. Philips et al. [31] reported that CUGBP1 binds to CUG sequences within splicing enhancers located downstream of the cTNT exon 5 and increases inclusion of this alternative exon in DM1. Subsequent studies showed that CUGBP1 promotes the embryonic splice form of both IR [30] and CIC-1 [26], which are predominant in DM1. CUGBP1 induces skipping of IR exon 11 by binding to both an exonic splicing enhancer on exon 11 and another intronic splicing enhancer on the upstream intron [30,41]. Interestingly, in these three examples (cTNT, IR and CIC-1), when CUGBP1 induces inclusion of an exon, MBNL1 increases exclusion of the same exon and vice versa. Kalsotra et al. [42] performed a large screen to identify alternative splicing events regulated during mouse heart development and identified CUGBP1 and MBNL1 as regulators of more than half of the splicing transitions tested. Many of these developmentally regulated splicing events are modulated

**Figure 2 | The levels of MBNL1 and CUGBP1 in the nucleus control a subset of developmentally regulated splicing events that are reversed in DM1**

In the embryonic stage, MBNL1 nuclear levels are low and CUGBP1 levels are high. During development, MBNL1 nuclear levels increase while CUGBP1 levels decrease, inducing an embryonic-to-adult transition of downstream splice targets (including IR exon 11, CIC-1 exons containing stop codons and cTNT exon 5). In DM1, MBNL1 is sequestered to CUG repeats, resulting in a decrease of functional MBNL1, while CUGBP1 levels are increased due to phosphorylation and stabilization. This simulates the embryonic condition and enhances expression of embryonic isoforms in adults, resulting in multiple disease symptoms.



exclusively by CUGBP1 or MBNL1, but all events regulated by both proteins exhibit antagonistic responses [42]. In addition, while MBNL1 nuclear levels increase during development, CUGBP1 nuclear levels decrease [22,42], supporting the hypothesis that the level and localization of these two proteins control a fetal to adult splicing transition, which is reversed in DM1 tissues (Figure 2).

CUGBP1 protein steady-state levels are increased in DM1 muscle [31] and heart tissue [43]. But does up-regulation of CUGBP1 contribute to DM1 pathogenesis? Two different transgenic mice overexpressing CUGBP1 in muscle were generated and both displayed muscle abnormalities similar to DM1 [44,45]. One mouse model exhibited splicing defects, providing further evidence that CUGBP1 contributes to mis-regulated splicing in DM1 [44]. The other model revealed another role of CUGBP1 as a regulator of translation. CUGBP1 interacts with the mRNA transcript of MEF2A (myocyte enhancer factor 2A), a transcription factor involved in myogenesis, and increases its translation [45]. MEF2A levels are increased in CUGBP1 transgenic mice and DM1 skeletal muscle tissues, which is proposed to contribute to delayed myogenesis [45]. Another known translational target of CUGBP1 is p21, which could cause impaired cell cycle withdrawal in DM1 muscle cells [46].

Besides regulating splicing and translation, CUGBP1 also plays a role in mRNA decay. Moraes et al. [47] reported that CUGBP1 binds to AREs (AU-rich elements) in *c-fos* and

TNF $\alpha$  (tumour necrosis factor  $\alpha$ ), followed by recruitment of the polyA-specific ribonuclease, which deadenylates and destabilizes the transcripts. Furthermore, TNF mRNA is stabilized in cells overexpressing the *DMPK* 3'-UTR containing expanded CUG repeats [48]. In addition to binding to AREs, CUGBP1 mediates mRNA decay of short-lived transcripts by interaction with GU-rich elements [49]. These studies reveal putative targets of CUGBP1 that may also contribute to DM1.

Several recent DM1 mouse models, all with repeats located within the *DMPK* 3'-UTR, exhibit increased CUGBP1 levels. Orenge et al. [14] generated inducible bitransgenic mice with muscle-specific expression of 960 CUG repeats that display many features of DM1 including RNA foci, myotonia, splicing defects, and importantly, increased CUGBP1 and muscle wasting. A heart specific mouse model expressing the same construct developed DM1-like cardiac conduction and contraction defects, and displayed elevated CUGBP1 levels within only 6 h after induction of the repeats [15]. Furthermore, CUGBP1 was up-regulated specifically in the nuclei in which RNA foci were detected [15]. This provides evidence that elevated CUGBP1 is a primary response to the mutant *DMPK* transcript. Mahadevan et al. [50] generated transgenic mice expressing the *DMPK* 3'-UTR containing only five CUGs as part of a GFP (green fluorescent protein) transcript. Surprisingly, these mice develop DM1-like phenotype and CUGBP1 is up-regulated in skeletal muscle; however, there was no formation of RNA foci and no obvious redistribution of nuclear MBNL1, although depletion of MBNL1 on 'micro-foci' cannot be ruled out [50].

How does expression of the DM1 repeat mutation result in increased CUGBP1 protein steady-state level? Kuyumcu-Martinez et al. [51] found that this is due to PKC (protein kinase C)-mediated hyperphosphorylation. Expression of expanded CUG repeats in the *DMPK* 3'-UTR induces CUGBP1 phosphorylation and is dependent on PKC activation. Activation of PKC increases CUGBP1 protein stability and PKC directly phosphorylates CUGBP1 *in vitro*. Moreover, PKC is activated in heart tissues from DM1 patients and a DM1 mouse model [51]. Thus it is hypothesized that the mutant RNA activates the PKC signalling pathway through an unknown mechanism, which induces CUGBP1 hyperphosphorylation and stabilization (Figure 1).

## Relevance to other RNA gain-of-function disorders

RNA toxicity caused by expression of non-coding repeat expansions is now known to be operative in other diseases. Huntington's disease-like 2 is caused by expanded CTG repeats located in the juncophilin-3 gene. The repeats can be in the coding or UTR depending on the alternative splicing pattern [52]. The CTG repeats form RNA foci that include MBNL1, similar to what is observed in DM1 [53]. MBNL1 is also found within nuclear inclusions in neurons of patients with FXTAS (fragile X associated tremor/ataxia syndrome) [54]. FXTAS is another RNA dominant disorder caused by

CGG repeats in the *FMR1* (fragile X mental retardation 1) gene 5'-UTR. This raises the possibility that sequestration of MBNL1 is a common pathogenic mechanism. Using a *Drosophila* model, Sofola et al. [55] further identified CUGBP1 as a suppressor of neurodegeneration induced by 90 CGG repeats in the *FMR1* 5'-UTR. The role of CUGBP1 in FXTAS is not yet understood. Most forms of SCA are caused by CAG expansions within coding regions, leading to a polyglutamine gain-of-function; however, several forms of SCA are caused by non-coding repeat expansions, including SCA8, SCA10 and SCA12 [25]. Interestingly, evidence from a *Drosophila* model indicates that, while CAG repeats of the SCA3 gene encode toxic protein, the RNA itself still contributes to the neurodegeneration phenotype [56].

## Conclusions

DM1 is one of the best characterized RNA dominant diseases. The pathogenic mechanisms involve protein sequestration, stimulation of signalling pathways, disruption of alternative splicing, mRNA translation and possibly mRNA stability. Understanding the mechanisms of this disease not only promotes the development of potential therapies but also provides insight for other diseases that share a common pathogenic mechanism.

## Funding

Work in our laboratory is supported by the National Institutes of Health and the Muscular Dystrophy Association.

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Received 6 July 2009  
doi:10.1042/BST0371281