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Molecular mechanisms of muscle atrophy in myotonic dystrophies

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Abstract

Myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) are multisystemic diseases that primarily affect skeletal muscle, causing myotonia, muscle atrophy, and muscle weakness. DM1 and DM2 pathologies are caused by expansion of CTG and CCTG repeats in non-coding regions of the genes encoding myotonic dystrophy protein kinase (DMPK) and Zinc finger protein 9 (ZNF9) respectively. These expansions cause DM pathologies through accumulation of mutant RNAs that alter RNA metabolism in patients' tissues by targeting RNA-binding proteins such as CUG-binding protein 1 (CUGBP1) and Muscle blind-like protein 1 (MBNL1). Despite overwhelming evidence showing the critical role of RNA-binding proteins in DM1 and DM2 pathologies, the downstream pathways by which these RNA-binding proteins cause muscle wasting and muscle weakness are not well understood. This review discusses the molecular pathways by which DM1 and DM2 mutations might cause muscle atrophy and describes progress towards the development of therapeutic interventions for muscle wasting and weakness in DM1 and DM2.

Keywords

Myotonic Dystrophy type 1; Myotonic Dystrophy type 2; CUG/CCUG repeats; muscle atrophy; therapeutic approaches

1. Introduction

Myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2) are complex multisystemic diseases that affect many tissues but primarily cause defects in skeletal muscle (Harper 2005; Ricker et al., 1995). In both diseases the severity of the clinical manifestations, including skeletal muscle pathology, varies between patients. There are four main clinical forms of DM1 that differ in the severity of the muscle phenotype and the age of onset. The most severe form of DM1 is a congenital form that affects newborn children. Skeletal muscle of patients with congenital DM1 shows a delay in development with severe muscle weakness, characterized by reduced muscle tone (hypotonia) (Amack and Mahadevan, 2004; Reardon et al., 1993). The childhood-onset form of DM1 is characterized by cognitive and behavioral abnormalities. Patients with the asymptomatic form of DM1 do

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not have muscle symptoms, whereas patients with adult onset of DM1 develop a progressive myotonia, muscle wasting, and weakness.

DM2 is a late-onset disease that mostly affects proximal muscles, causing muscle weakness, muscle pain, and myotonia. There is no congenital form of DM2. Whereas DM1 is characterized by severe muscle atrophy, DM2 is a much milder disease. Both diseases are associated with cardiac defects, endocrine abnormalities, neurological dysfunctions, and cataracts (Harper, 2001; Ricker et al., 1995). Histological studies of skeletal muscle sections from patients with DM1 and DM2 show atrophic fibers, variability of myofiber size, ring fibers, and an increase in central nuclei. Different fiber types are atrophic in DM1 and DM2: there is an increase in atrophic type 1 (slow twitch) fibers in DM1, whereas type 2 fibers are atrophic in DM2 (Vihola et al., 2003).

DM1 is caused by a polymorphic CTG triplet repeat expansion within the 3' untranslated region (UTR) of the *DMPK* gene on chromosome 19q (Brook et al., 1992; Fu et al., 1992). In unaffected individuals these CTG expansions vary in length from 5 to 35 repeats, but in patients with DM1 the number of CTG repeats is increased to the range of 50 to several thousand repeats. The severity of the clinical phenotype in different patients with DM1 is dependent on the number of CTG repeats.

DM2 is caused by unstable expansion of CCTG tetranucleotide repeats in intron 1 of the gene encoding ZNF9 (also known as Cellular Nucleic Acid-binding Protein, CNBP) (Liquori et al., 2001). In unaffected individuals, the length of these CCTG repeats does not exceed 26 repeats; however, patients with DM2 have very long expansions of up to several thousand repeats. In contrast to the positive correlation between phenotype severity and the number of CTG repeats in patients with DM1, in DM2 there is no clear relationship between the length of the CCTG repeats and the severity of disease. Although both DM1 and DM2 are multisystemic diseases, the most devastating symptoms in both diseases that significantly affect the quality of life are skeletal muscle atrophy and muscle weakness.

Studies of the molecular mechanisms by which polymorphic expansions of CTG and CCTG repeats cause the muscle pathology of DM1 and DM2 revealed the critical role of RNA CUG and CCUG repeats. Because the CTG and CCTG expansions are both located in non-coding regions of the disease genes they do not disrupt the major protein product. However, CTG repeat expansion might produce short peptides through AUG-independent translation (Zu et al., 2011). Importantly, the mutant RNAs containing CUG and CCUG repeats have increased stability and reduced turnover (Jones et al., 2011), which is likely responsible for the accumulation of non-degraded mutant RNA within patients' cells. It is well established that the accumulation of these mutant RNAs is toxic to RNA metabolism because the CUG and CCUG repeats bind RNA-binding proteins, thus interfering with their normal activities (Schoser and Timchenko, 2010).

The major RNA-binding proteins that mediate the pathologic effects of mutant CUG and CCUG repeats are MBNL1 and CUGBP1 (a member of the CUGBP and Embryonic Lethal abnormal vision-like Family of proteins, CELF) (Mankodi et al., 2000; Miller et al., 2000; Philips et al., 1998; Timchenko 1999; Timchenko et al., 1996a; Timchenko et al., 1996b). Several reports have suggested that ZNF9, a protein with DNA- and RNA-binding activities (Calcaterra et al., 2010), is also involved in DM2 pathology (Chen et al., 2007; Huichalaf et al., 2009; Raheem et al., 2010; Sammons et al., 2010). Recent data showed that the mutant CUG repeats might impair RNA-binding proteins (such as CUGBP1) through disruption of signaling pathways mediated by PKC and GSK3 β kinases (Jones et al., 2012; Kuyumcu-Martinez et al., 2007). Alteration of activities of RNA-binding proteins in DM leads to myotonia, muscle atrophy, and weakness in skeletal muscle and has toxic effects in other

tissues. In this review, we will focus on the molecular pathways associated with muscle atrophy in DM1 and in DM2 and discuss how molecular advances have been translated into the development of therapeutic approaches for DM.

2. Muscle atrophy in DM1 is caused by expanded RNA CUG repeats

Several mouse models have been generated for examination of the molecular basis of DM1 pathology. A major group of DM1 mouse models addresses the role of the accumulation of long CUG repeats in DM1 muscle pathology (Mankodi et al., 2000; Orengo et al., 2008; Seznec et al., 2001). In these mouse models, CUG repeats are expressed within the entire *DMPK* gene (Seznec et al., 2001), in the 3' UTR of *DMPK* mRNA (Orengo et al., 2008), or in the 3' UTR of mRNA unrelated to *DMPK* (Mankodi et al., 2000). The second group of DM1 mouse models includes models designed to elucidate the role of RNA-binding proteins targeted by mutant CUG repeats in DM1 (CUGBP1 and MBNL1) through overexpression of CUGBP1 and deletion of MBNL1 (Ho et al., 2005; Kanadia et al., 2003; Timchenko et al., 2004; Ward et al., 2010). The first transgenic mouse model that demonstrated the crucial role of CUG RNA in DM1 muscle pathology expressed an array of pure CUG repeats (250 repeats) in the 3' UTR of the gene encoding skeletal muscle actin (HSA^{LR} mice) (Mankodi et al., 2000). These mice developed myotonia and skeletal muscle myopathy, which was characterized by an increase in central nuclei, variability of myofiber size, and nuclear chains, similar to the muscle histopathology in patients with DM1. Whereas initial studies did not reveal overt muscle atrophy in adult (up to 6 months of age) HSA^{LR} mice (Mankodi et al., 2000), recent analysis showed that the total number of myofibers is reduced in *gastrocnemius* of six-month-old HSA^{LR} mice in at least some mouse lines of this model (e.g., line LR20b) (Jones et al., 2012). The reduction in the total number of fibers in *gastrocnemius* of six-month-old HSA^{LR} mice is accompanied by a strong variability in myofiber size with an increase in both small and enlarged fibers (Jones et al., 2012). This analysis showed that the expression of long CUG repeats in DM1 causes muscle atrophy. Consistent with the reduction in fiber number, HSA^{LR} mice are characterized by reduced grip strength (Jones et al., 2012), mimicking the reduced handgrip strength in patients with DM1 (Tang et al., 2012). It is not yet known whether fiber loss in HSA^{LR} *gastrocnemius* progresses with age. It is important to note that the number of fibers in *tibialis anterior* in the six-month-old HSA^{LR} mice was not changed (Jones et al., 2012). Moreover, approximately 20% of HSA^{LR} mice have normal grip strength (Jones et al., 2012). Other reports show that muscle force is impaired in extensor digitorum longus of the five-month-old HSA^{LR} mice, but not in soleus or diaphragm (Moyer et al., 2011). Elucidation of the reasons for the variability of the symptoms in HSA^{LR} muscle requires further investigations. It would be important to characterize phenotype in all muscle groups in these mice using the same approaches. The length of CTG repeats and the levels of expression of CUG RNA in different muscle groups in HSA^{LR} mice should be determined to correlate muscle phenotype with the length of CTG repeats and with the levels of CUG-containing transcripts.

A similar effect of mutant CUG repeats on muscle pathology was observed in a DM1 mouse model that expresses the entire human *DMPK* gene containing an array of 300 expanded CTG repeats (DM300 mice) (Seznec et al., 2001). Skeletal muscle of these mice is characterized by myotonia, increased number of fibers with central nuclei, increased variability in fiber size, and focal areas of regeneration-degeneration that express the neonatal myosin heavy chain. In addition to these abnormalities, DM300 transgenic mice have an increased number of atrophic fibers with specific atrophy of type 1 fibers (Seznec et al., 2001). Consistent with the muscle atrophy, whole body weight was reduced in these mice even at a young age (Seznec et al., 2001). The same mice with an increased length of CTG expansion (DM550) developed progressive age-dependent muscle weakness and wasting associated with reduced muscle mass and fiber diameter (Vignaud et al., 2010).

The most striking muscle wasting was observed in the DM1 inducible mouse model expressing a mutant 3' UTR of *DMPK* containing a long CUG expansion (960 repeats) under the control of a skeletal muscle promoter (Orengo et al., 2008). In addition to myopathic abnormalities typical of DM1 muscle pathology, expression of the mutant 3' UTR of *DMPK* in these mice caused severe muscle wasting that reduced muscle size. Notably, the muscle wasting in these mice was progressive during the course of expression of the mutant *DMPK* 3' UTR (Orengo et al., 2008). Fiber loss in the inducible DM1 mouse model was accompanied by an increase in small-sized fibers, fibrosis, myofiber degeneration, and a reduction in performance on the treadmill (Orengo et al., 2008).

Thus, analyses of DM1 mouse models showed that progressive muscle wasting in DM1 mouse models is caused by CTG repeats. However, the severity of muscle pathology in different mouse models of DM1 varies. This variability is likely dependent on the length of CTG expansions, the genomic environment of CTG repeats, the nature of repeats (pure or interrupted) and on the efficiency of expression of the mutant CUG-containing transcripts. Additional factors, such as accumulation of mutant antisense transcripts, might also contribute to the severity of muscle pathology in mouse models of DM1 (Huguet et al., 2012).

2.1. The role of abnormal splicing in muscle wasting and muscle weakness in DM1

Although it is well established that expanded CUG repeats are responsible for the myofiber loss in DM1, the downstream pathways by which the mutant CUG repeats cause muscle wasting are not well understood. Because the mutant CUG repeats mainly misregulate two RNA-binding proteins, CUGBP1 and MBNL1 (Miller et al., 2000; Philips et al., 1998; Timchenko et al., 1996a; Timchenko et al., 1996b), it is likely that abnormal functioning of these proteins causes the muscle atrophy in DM1 (Figure 1). Mouse models with increased expression levels of CUGBP1 in the whole body or specifically in skeletal muscle revealed the crucial role of CUGBP1 in muscle development and function. Elevation of CUGBP1 expression in traditional transgenic mouse models interferes with muscle development, causing a delay in myogenesis consistent with the muscle abnormalities in congenital DM1 (Ho et al., 2005; Timchenko et al., 2004). Adult transgenic mice expressing elevated levels of CUGBP1 develop a dystrophic phenotype (Timchenko et al., 2004).

The muscle of mice with deleted MBNL1 shows myotonia, split fibers, and an increase in central nuclei (Kanadia et al., 2003). Although the previous studies showed that the main function of MBNL1 is regulation of splicing (Kanadia et al., 2003; Lin et al., 2006), recent reports showed that MBNL1 is also involved in control of destabilization of a broad spectrum of mRNAs (Masuda et al., 2012), in miR-1 processing (Rau et al., 2011), and in regulating localization of mRNAs (Wang et al., 2012). CUGBP1 is a multifunctional protein that functions in the nuclei and cytoplasm and regulates splicing, stability, and translation of RNAs (Charlet et al., 2002; Lee et al., 2010; Moraes et al., 2006; Paillard et al., 1998; Savkur et al., 2001; Timchenko et al., 2005; Timchenko et al., 2006; Vlasova et al., 2008; Zhang et al., 2008). The mutant CUG repeats have opposite effects on the splicing activities of these two proteins; the splicing activity of MBNL1 is reduced by the mutant CUG repeats due to sequestration of MBNL1 by the nuclear CUG aggregates (Miller et al., 2000) whereas CUG repeats increase protein levels of CUGBP1 as a result of its increased stability (Mahadevan et al., 2006; Savkur et al., 2001; Timchenko et al., 2001). The toxic effect of the mutant CUG repeats on the activity of CUGBP1 is complex and involves phosphorylation of CUGBP1 at Ser302 by a GSK3 β -cyclin D3/cdk4 pathway (Huichalaf et al., 2010; Jones et al., 2012). As the result of this phosphorylation, CUGBP1 exists in two states, an un-phosphorylated at Ser302 form and an isoform that is phosphorylated at Ser302. Levels of both forms of CUGBP1 are elevated in DM1 muscle. It has been shown that the phosphorylation of CUGBP1 at Ser302 regulates CUGBP1 translational activity

(Timchenko et al., 2006); however, the role of this phosphorylation on other activities of CUGBP1, such as regulation of RNA stability and splicing, remains to be examined. CUGBP1 is also phosphorylated by Akt (Huichalaf et al., 2010) and PKC kinase (Kuyumcu-Martinez et al., 2007). Phosphorylation of CUGBP1 by Akt regulates the nucleus-cytoplasm distribution of CUGBP1 (Huichalaf et al., 2010) whereas phosphorylation by PKC stabilizes CUGBP1 protein in DM1 (Kuyumcu-Martinez et al., 2007).

MBNL1 and CUGBP1 have antagonistic splicing activities, and function to regulate spliced isoforms during normal skeletal muscle and heart development (Ranum and Cooper, 2006). In DM1, a reduction in MBNL1 splicing activity and increase in CUGBP1 splicing activity results in accumulation of embryonic-specific transcripts and their protein products in adult muscle, affecting muscle function and leading to muscle atrophy (Figure 1). Several mRNAs that play a critical role in normal muscle function show abnormal splicing patterns in DM1, including mRNAs encoding a skeletal muscle chloride ion channel 1 (Clcn1) (Charlet et al., 2002; Mankodi et al., 2002), sarcoplasmic/endoplasmic reticulum Ca^{2+} ATP-ase (SERCA1) (Kimura et al., 2005), a protein localized to the Z line (Cypher) (Kanadia et al., 2006), a protein involved in the T-tubule formation (BIN1) (Fugier et al., 2011), Ca^{2+} release channel or Ryanodine Receptor 1 (RyR1) (Kimura et al., 2005), and L-type Ca^{2+} channel and voltage sensor (Cav1.1) (Tang et al., 2011). Mis-regulation of SERCA1 and RyR1 in DM1 might affect Ca^{2+} homeostasis, leading to the activation of Ca^{2+} -dependent proteases such as calpain and causing muscle atrophy. Abnormalities of BIN1 splicing might disrupt formation of T-tubules resulting in muscle under-development and weakness. The Cav1.1 channel plays a crucial role in the regulation of excitation-contraction coupling (Tang et al., 2002). Thornton's group found that mis-splicing of Cav1.1 serves as a marker of muscle weakness in patients with DM1 and DM2 because the severity of abnormalities in Cav1.1 splicing correlates with muscle weakness in patients with DM (Tang et al., 2011). It appears that both CUGBP1 and MBNL1 have the ability to regulate splicing of Cav1.1 (Tang et al., 2011) although the specific contribution of these splicing activities to muscle wasting in DM1 remains to be determined. One difficulty associated with these studies is that CUGBP1 is a multi-functional protein; therefore, to determine the specific contribution of the splicing activity of CUGBP1 to muscle atrophy the cytoplasmic functions of CUGBP1 must be repressed. In addition, these two proteins regulate splicing of the same sub-set of mRNAs (Kalsotra et al., 2008).

Comparison of splicing patterns in the muscle of HSA^{LR} mice with those in mice with deleted MBNL1 shows similar splicing abnormalities (Osborne et al., 2009). However, *MBNL1* knock-out (KO) mice do not develop overt muscle wasting (Kanadia et al., 2003), whereas an inducible mouse model of DM1 expressing the 3' UTR of *DMPK* with 960 CUG repeats shows severe muscle atrophy (Orengo et al., 2008). Whereas there is a possibility that other members of MBNL family (MBNL2 or MBNL3) might compensate for the lack of MBNL1, a critical difference between *MBNL1* KO mice and CUG-inducible mice is that the *MBNL1* KO mice have normal levels of CUGBP1, whereas CUGBP1 expression is elevated in the inducible DM1 model (Orengo et al., 2008). It is possible that the elevated CUGBP1 is responsible for muscle wasting in the CUG-inducible DM1 mouse model (Orengo et al., 2008). Analysis of splicing events identified several mRNAs that showed abnormal splicing in this model but normal splicing patterns in *MBNL1* KO mice. These mRNAs include those encoding Ankyrin 2 (Ank2), F actin capping protein beta subunit (Capzb), and fragile X mental retardation syndrome-associated protein (Fxr1) (Orengo et al., 2008). Ank2 mediates binding of membrane proteins to the cytoskeleton and Capzb is associated with filament growth. Fxr1 is a member of the fragile X family of RNA-binding proteins that regulate RNA processing on several levels, including translation (Whitman et al., 2011). Deletion of Fxr1 in mice causes muscle loss (Mientjes et al., 2004);

therefore, abnormal splicing of Fxr1 mediated by CUGBP1 might reduce the synthesis of many proteins in DM1 cells, contributing to muscle atrophy.

In agreement with this suggestion, the CUGBP1 mouse model with tet-regulated elevation of CUGBP1 in skeletal muscle developed myofiber atrophy that was associated with the accumulation of small fibers, increased inflammatory infiltrates, and fiber degeneration (Ward et al., 2010). The accumulation of atrophic fibers in these mice is accompanied by a reduction in the weight of individual muscles and in whole body weight. Several mRNAs that are important for muscle function, including those for SERCA1 and RyR1 receptor, showed abnormal splicing patterns in these mice suggesting that increased expression of CUGBP1 might affect excitation-contraction coupling and thus reduce muscle strength (Ward et al., 2010). Consistent with these findings, the performance of mice on the treadmill is reduced upon up-regulation of CUGBP1 in skeletal muscle. Although mice with elevated CUGBP1 expression clearly develop muscle atrophy, the contribution of the splicing activity of CUGBP1 to muscle atrophy remains to be determined. A recent study using a mouse model that expresses a nuclear dominant-negative form of CUGBP1 showed that these mice have disrupted splicing without muscle atrophy (Berger et al., 2011). The muscle in these mice is characterized by an increase in slow twitch fibers, increased variability in fiber size, and a reduction in endomysial and perimysial spaces (Berger et al., 2011). The lack of muscle atrophy and weakness in these mice suggest that abnormal cytoplasmic functions of CUGBP1 (translation and RNA stability) might contribute to muscle atrophy in DM1.

2.2. The role of altered translational functions of CUGBP1 in muscle atrophy and weakness in DM1

As mentioned above, one abnormal feature of DM1 muscle is the elevated expression of CUGBP1 that is un-phosphorylated at Ser302. This isoform represses protein translation in stress granules (SGs) (Huichalaf et al., 2010); therefore, inhibition of protein synthesis in DM1 SGs might contribute to muscle wasting (Figure 1). Analysis of signaling pathways that regulate CUGBP1 phosphorylation at Ser302 showed that CUG repeats reduce phosphorylation of Ser302 through a reduction in cyclin D3, which activates cdk4 kinase (Salisbury et al., 2008). This reduction of cyclin D3 in DM1 muscle cells is due to elevated levels of active GSK3 β kinase (Jones et al., 2012), which has many substrates and phosphorylates cyclin D3 at Thr483, targeting it for degradation (Naderi et al., 2004). The impairment of the GSK3 β -cyclin D3-CUGBP1 pathway in HSA^{LR} muscle shows that the mutant CUG repeats are responsible for the elevated activity of GSK3 β in DM1 (Jones et al., 2012), although the underlying mechanism is not known. The significance of the GSK3 β -cyclin D3-CUGBP1 pathway in muscle pathology in DM1 was further demonstrated in experiments showing that inhibitors of GSK3 β corrected CUGBP1 translational activity and increased the number of activated myogenic satellite cells (Jones et al., 2012).

Quiescent satellite cells located beneath the basal lamina are usually activated in response to muscle injury or damage to repair and maintain myofibers. In damaged muscle, satellite cells proliferate and fuse with damaged fibers or form new fibers to replace degenerated fibers. In DM1 the efficiency of satellite cell proliferation is reduced (Bigot et al., 2009; Thornell et al., 2009). The diminished ability of satellite cells to proliferate might lead to reduced fiber regeneration, atrophy, and muscle weakness. It appears that correction of the GSK3 β -cyclin D3-CUGBP1 pathway in the 6-month-old HSA^{LR} mice by treatment with GSK3 β inhibitors might increase the number of myogenic satellite cells (Jones et al., 2012). This increase of myogenic satellite cells in HSA^{LR} muscle is accompanied by the reduction of muscle histopathology and improvement of the grip strength. It remains to investigate if the increase of myogenic satellite cells improves muscle regeneration in HSA^{LR} mice. Although correction of grip strength in HSA^{LR} mice by GSK3 β inhibitors correlates with

normalization of the cyclin D3-CUGBP1 pathway and correction of CUGBP1 translational function, the pathways by which GSK3 β inhibitors increase the number of activated myogenic satellite cells are not known.

Examination of CUGBP1 in HSA^{LR} mice is a subject of contradictory observations. The previous study showed no change of CUGBP1 levels in HSA^{LR} mice (Lin et al., 2006); however, a recent report indicated that CUGBP1 levels are increased in muscle of these mice (Jones et al., 2012). The reason for these discrepancies is not known and needs additional studies. It has been shown that the inhibitors of GSK3 β have a positive effect on the CUGBP1 activity in muscle of HSA^{LR} mice but whether the correction of GSK3 β “normalizes” CUGBP1 levels remains to be examined.

It has been shown that CUGBP1 also regulates RNA stability. In fact, a large number of mRNAs with binding sites for CUGBP1 within their 3' UTRs were identified in cultured cells, including mouse C2C12 myoblasts (Lee et al., 2010; Rattenbacher et al., 2010; Vlasova et al., 2008). Some of these mRNAs encode proteins that are important for muscle development and function. Therefore, it is expected that the mis-regulation of CUGBP1 in DM1 might also affect stability of mRNAs that contribute to the development of muscle atrophy in DM1.

3. The molecular mechanisms of muscle atrophy in DM2

Little is known about the molecular mechanisms of muscle atrophy in DM2. In contrast to DM1, the role of MBNL1 and CUGBP1 in muscle atrophy in DM2 has been poorly investigated. Because MBNL1 is sequestered by the mutant CCUG foci in DM2 cells (Mankodi et al., 2001), it is expected that the CCUG repeat expansions will have a greater inhibitory effect on MBNL1 splicing activity in DM2 than in DM1 because DM2 expansions are longer than DM1 expansions. However, muscle atrophy in DM2 muscle is milder than that in DM1, suggesting that MBNL1 sequestration and a reduction of MBNL1 splicing activity in DM2 cells might play only a partial role in DM2 muscle atrophy. The contribution of CUGBP1 to DM2 also requires additional investigation because some patients with DM2 show increased expression of CUGBP1 (Salisbury et al., 2009), whereas other patients have normal levels of CUGBP1 (Lin et al., 2006; Pelletier et al., 2009). Analysis of the cellular toxicity of the mutant CCUG repeats using tet-regulated cell culture models showed that mutant CCUG repeats over an extended range elevate CUGBP1 levels, but at a slower rate than mutant CUG repeats (Jones et al., 2011). Surprisingly, a high level of short CCUG repeats elevated CUGBP1 levels in DM2 cell culture models more strongly than long CCUG repeats (Jones et al., 2011).

The status of CUGBP1 translational activity in DM2 is also not well understood. Compared with DM1, in which elevated expression of GSK3 β and a reduction in cyclin D3 activity increase the amount of CUGBP1 that is un-phosphorylated at Ser302, the pathways regulating CUGBP1 activity in DM2 are unknown. The role of GSK3 β -cyclin D3 signaling in DM2 also remains to be examined. Our group showed that levels of the translational targets of CUGBP1 (C/EBP β and MEF2A) are increased in DM2 muscle biopsies (Salisbury et al., 2009), suggesting that CCUG repeats elevate expression of the active, phosphorylated at Ser302 isoform of CUGBP1. However, there are no studies addressing the levels of un-phosphorylated CUGBP1 in DM2 muscle cells. Comparison of DM1 and DM2 myoblast cell culture models showed that levels of active CUGBP1-eIF2 complexes are reduced during differentiation of DM1 myoblasts, whereas the amounts of these complexes are normal in DM2 myotubes (Schoser and Timchenko, 2010). This result suggests that the regulation of CUGBP1 activity in DM1 and DM2 muscles might be different. The detailed

mechanisms underlying the misregulation of CUGBP1 and MBNL1 in DM2 muscle cells and their possible connection with muscle atrophy remain to be elucidated.

3.1 The role of ZNF9-mediated reduction of the rate of protein synthesis in muscle atrophy in DM2

Given the expansion of CCTG tetranucleotide repeats in the *ZNF9* gene, ZNF9 protein is a potential candidate for involvement in muscle atrophy and weakness in DM2. Two recent reports showed that ZNF9 levels are reduced in DM2 muscle biopsies (Salisbury et al., 2009; Raheem et al., 2010), although some patients with DM2 have normal levels of ZNF9 (Botta et al., 2006; Margolis et al., 2006). The mechanism by which the mutant CCUG repeats decrease ZNF9 expression is not well understood, but it has been shown that ZNF9 plays a key role in the translational control of mRNAs containing 5'-terminal oligopyrimidine (5' TOP) tracts (Pellizzoni et al., 1997; Huichalaf et al., 2009). The TOP mRNAs encode proteins of the translational apparatus, including ribosomal proteins and elongation factors (Meyuhas, 2000). DM2 muscle biopsies have shown reduced levels of several TOP proteins leading to a reduction in the rate of global protein synthesis (Huichalaf et al., 2009), which might explain the muscle atrophy in DM2 (Figure 2). This hypothesis is supported by the recent identification of many mRNA targets that encode proteins involved in protein synthesis bound to ZNF9 (Sammons et al., 2011; Scherrer et al., 2011). Moreover, some of the mRNAs that are bound by ZNF9 encode proteins important for muscle function (Scherrer et al., 2011). The muscle phenotype in *ZNF9* KO mice, which includes myofiber size variability and weakness, mimics DM2 muscle pathology and supports the role of ZNF9 in muscle function (Chen et al., 2007). Thus, ZNF9 is a reasonable candidate for involvement in muscle atrophy in DM2, although this should be confirmed in DM2 mouse models that express a non-coding array of RNA CCUG repeats.

3.2. The role of proteasome dysfunction in muscle atrophy in patients with DM2

Muscle atrophy can occur due to a misbalance between protein synthesis and protein degradation. It has been shown that the stability of short-lived proteins such as c-myc and p21 that are degraded by the ubiquitin-proteasome system (UPS) is increased in DM2 myoblasts (Salisbury et al., 2009). This suggests that proteasome activity in DM2 muscle cells is affected, possibly as a result of the binding of RNA CCUG repeats to multiprotein complexes containing the 20S proteasome (Salisbury et al., 2009). Experiments in tet-regulated CCUG-expressing cell models showed that the levels of p21 are increased by expression of mutant CCUG repeats, but only at later stages of CCUG repeat expression (Jones et al., 2011). Examination of the time-line of molecular and cellular toxic events caused by expression of the CCUG repeats showed that accumulation of RNA CCUG foci, an increase in CUGBP1, and a reduction in ZNF9 are early events that occur 1–7 hours after induction of CCUG transcription; however, the elevation of p21, a target of the UPS, occurs 17 hours after induction of CCUG transcription (Jones et al., 2011). These data suggest that mis-regulation of several molecular and cellular pathways caused by CCUG repeats might lead to dysfunction of the UPS system, which contributes to muscle atrophy (Figure 2). Proteomic analysis of DM2 myotubes also indicated dysfunction of protein degradation (Rusconi et al., 2010).

It is important to note that protein degradation might also play a role in the development of atrophy in DM1. Recent studies of muscle atrophy in CTG550 mice showed increased trypsin-like activity of proteasome in muscle of these mice that correlated with the development of muscle wasting (Vignaud et al., 2010). In addition, expression of a protein Fbxo32, which degrades MyoD and eIF3-f in skeletal muscle (Lagrand-Cantaloube et al., 2008; Tintignac et al., 2005), was also increased in muscle of CTG550 mice (Vignaud et al., 2010).

4. Development of therapeutic approaches to the correction of muscle atrophy and weakness in DM

Identification of the toxic role of mutant CUG repeats in DM1 opened the possibility of developing therapies based on reduction of the toxicity of mutant *DMPK* mRNA. Use of the inducible DM1 mouse model, in which tet-dependent expression of high levels of the *DMPK* 3' UTR caused symptoms of DM1 whereas withdrawal of doxycycline reduced expression of the 3' UTR of *DMPK* and diminished the myotonia and myopathy, showed that DM1 muscle pathology could be reversed by degradation of CUG repeats (Mahadevan et al., 2006).

Two major approaches have been used to reduce the toxicity of the mutant CUG repeats in pre-clinical studies of DM1. The first approach is based on reduction of the levels of mutant CUG RNA by antisense oligonucleotides (ASOs) whereas the second involves techniques that “normalize” the activities of the RNA-binding proteins affected by the mutant CUG repeats, such as MBNL1 and CUGBP1.

Initial approaches to correct the muscle pathology in DM1 mouse models were based on ASOs containing modified CAG repeats. Use of morpholino CAG ASOs in HSA^{LR} mice reduced the number of CUG foci, corrected MBNL1 splicing activity, and reduced myotonia (Wheeler et al., 2009). 2'-Ome-phosphorothioate CAG ASOs also reduced CUG foci and corrected splicing in HSA^{LR} and DM500 mouse models (Mulders et al., 2009). Modified CAG ASOs that increase degradation of the mutant RNA by RNAase H reduced the levels of mutant CUG repeats and corrected splicing abnormalities in a DM1 mouse model (Lee et al., 2012). However, these ASOs themselves caused muscle damage, therefore further studies are needed to examine ASO toxicity (Lee et al., 2012).

A recent study showed that an RNase H-active ASO targeting the mutant RNA outside of the CUG repeat sequence reduced the expression levels of the mutant RNA in a DM1 mouse model (HSA^{LR} mice) without obvious side effects (Wheeler et al., 2012). Importantly, HSA^{LR} mice that were treated at a young age (2 months) showed an improvement in muscle phenotype, including a reduction of muscle-fiber size variability, 1 year after the final treatment (Wheeler et al., 2012). This study indicates that ASOs targeting regions outside the CUG repeat expansion might be beneficial for degradation of the mutant RNA in DM1 (Figure 3).

Other therapeutic approaches are focused on the correction of MBNL1 and CUGBP1 activities. Delivery of MBNL1 to HSA^{LR} mice using a recombinant adeno-associated viral vector reduced myotonia but had no positive effect on muscle histopathology (Kanadia et al., 2006). Correction of CUGBP1 levels by inhibitors of PKC improved cardiac pathology in the mouse model of cardiac dysfunction in DM1 (Wang et al., 2009). Moreover, the repressed translational activity of CUGBP1 in DM1 can be corrected by inhibition of GSK3 β (Jones et al., 2012) (Figure 3). GSK3 β inhibitors such as lithium and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) de-repress CUGBP1 translational activity in HSA^{LR} muscle by increasing phosphorylation of CUGBP1 at Ser302, leading to a reduction in myotonia, a reduction in myofibers with central nuclei, and improvement of grip strength (Jones et al., 2012). Importantly, short-term treatment (three times a week for 2 weeks) of young (6-week-old) HSA^{LR} mice with TDZD-8 had a positive effect on grip strength at 3 months of age (Jones et al., 2012). This observation suggests that correction of CUGBP1 activity during the period of time when muscle is not significantly affected by the mutant CUG repeats can delay the progression of DM1 pathology. This result agrees with findings from Dr. Thornton's lab that short-term treatment (twice a week for 4 weeks) of 2-month-old HSA^{LR} mice with ASO resulted in the correction of the age-dependent

myopathic changes, as evidenced by reduced frequency of central nuclei, improved muscle-fibre diameter and reduced myotonia in 1-year-old mice (Wheeler et al., 2012). In addition, treatment of HSA^{LR} mice with GSK3 β inhibitors increased the number of activated myogenic satellite cells (Jones et al., 2012), suggesting that the increase of satellite cells in myofibers prior to development of muscle atrophy might have the beneficial effect of delaying muscle pathology. Because mutant CUG repeats elevate expression of GSK3 β , thus reducing CUGBP1 activity, it is expected that degradation of the mutant CUG repeats in young HSA^{LR} mice, treated with ASOs, might improve CUGBP1 activity and increase the number of activated myogenic satellite cells, thereby maintaining normal function of myofibers during the animal's lifespan. Thus, degradation of mutant RNA containing CUG repeats or correction of CUGBP1 activity reduces muscle pathology in the mouse model of DM1.

5. Conclusions

1. Current evidence suggests that mutant CUG repeats are responsible for muscle atrophy in DM1. Degradation of the mutant CUG repeats in DM1 mouse models is sufficient to correct muscle histopathology and other muscle symptoms of DM1.
2. Additional studies are needed to determine the contribution of MBNL1 and CUGBP1 to the development of muscle atrophy and weakness in DM1. Current data show that up-regulation of CUGBP1 is sufficient to cause muscle atrophy *in vivo*. Although correction of the mis-splicing of RNAs that are targets of MBNL1 correlates with the correction of muscle histopathology in a DM1 mouse model (HSA^{LR}), correction of free levels of MBNL1 in the same mouse model reduced myotonia but was not sufficient to improve muscle histopathology. These data suggest that mis-regulation of CUGBP1 plays a key role in the development of muscle atrophy in DM1.
3. Because CUGBP1 has many functions (splicing, translation, and stability), the role of each individual activity in the development of muscle atrophy in DM1 needs to be determined. Whereas correction of myotonia in DM1 might be achieved by increased MBNL1 activity, correction of CUGBP1 activity using GSK3 β inhibitors increases grip strength, reduces myotonia and reduces muscle histopathology. Although the mechanisms by which GSK3 β is increased in DM1 remain to be addressed, the benefits of GSK3 β inhibitors in the reduction of muscle pathology in the mouse model of DM1 suggest that these inhibitors might be used in DM1 clinical studies.
4. The majority of studies in the DM field are focused on the molecular mechanisms of DM1 and the development of therapeutic approaches for DM1, whereas studies on DM2 are limited. The roles of CUGBP1 and MBNL1 in the muscle pathology of DM2 need to be investigated in detail. ZNF9 is a reasonable candidate player in the development of DM2 atrophy at the level of protein translation; however, its role in DM2 pathogenesis needs to be confirmed in a larger number of patients with DM2 and in DM2 mouse models expressing mutant CCUG repeats.
5. Misregulation of protein degradation in DM2 is an attractive pathway for the development of muscle atrophy that needs to be further studied using DM2 mouse models expressing RNA CCUG repeats.

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Figure 1. Model showing molecular pathways by which mutant *DMPK* mRNA might cause muscle atrophy and muscle weakness in DM1

The mutant CUG repeats reduce activity of MBNL1, increase total levels of CUGBP1, and increase levels of CUGBP1 that is not phosphorylated at S302, which represses protein translation in stress granules of DM1 cells. As a consequence, splicing and translation of mRNAs are mis-regulated in DM1 muscle, resulting in muscle atrophy and weakness.

Figure 2. A hypothetical model showing the proteins and pathways by which mutant CCUG repeats might cause muscle atrophy in DM2 muscle

Intron 1 of the *ZNF9* gene containing expanded CCTG repeats is shown in the blue box, other introns are shown in green, and exons are shown in black. ZNF9 controls translation of TOP proteins involved in the translational machinery. Reduced levels of ZNF9 in patients with DM2 might cause a reduction in the expression of TOP proteins, leading to a reduction in the rate of global protein synthesis and resulting in muscle atrophy. Dysfunction of proteasomes, increased expression of CUGBP1, and a reduction in MBNL1 activity might also contribute to muscle atrophy in DM2.

Figure 3. Model showing two therapeutic approaches to correct muscle pathology in DM1
 In the first approach, ASOs reduce the level of mutant *DMPK* mRNA leading to a correction of muscle pathology. The second approach uses GSK3 β inhibitors to normalize GSK3 β , which is activated by the mutant CUG repeats. These inhibitors correct the activity of CUGBP1 and increase the number of myogenic satellite cells. The inhibitors of GSK3 β reduce myotonia, increase grip strength, and reduce DM1 muscle histopathology.