Heart-specific overexpression of CUGBP1 reproduces functional and molecular abnormalities of myotonic dystrophy type 1

Misha Koshelev1,†, Satyam Sarma2,3,‡, Roger E. Price5, Xander H.T. Wehrens2,3 and Thomas A. Cooper1,4,*

1Department of Pathology, 2Department of Molecular Physiology and Biophysics, 3Department of Medicine (Cardiology), 4Department of Molecular and Cellular Biology and 5Center for Comparative Medicine, Baylor College of Medicine, Houston, TX 77030, USA

Received November 1, 2009; Revised and Accepted December 21, 2009

Myotonic dystrophy type 1 (DM1) is caused by a CTG expansion within the 3'-untranslated region of the DMPK gene. The predominant mechanism of pathogenesis is a toxic gain of function of CUG repeat containing RNA transcribed from the expanded allele. The molecular mechanisms by which the RNA containing expanded repeats produce pathogenic effects include: sequestration of muscleblind-like 1 (MBNL1) protein and up-regulation of CUG binding protein 1 (CUGBP1). MBNL1 and CUGBP1 are RNA binding proteins that regulate alternative splicing transitions during development. Altered functions of these proteins in DM1 lead to misregulated splicing of their target genes, resulting in several features of the disease. The role of MBNL1 depletion in DM1 is well established through a mouse knock-out model that reproduces many disease features. Here we directly test the hypothesis that CUGBP1 up-regulation also contributes to manifestations of DM1. Using tetracycline-inducible CUGBP1 and heart-specific reverse tetracycline trans-activator transgenes, we expressed human CUGBP1 in adult mouse heart. Our results demonstrate that up-regulation of CUGBP1 is sufficient to reproduce molecular, histopathological and functional changes observed in a previously described DM1 mouse model that expresses expanded CUG RNA repeats as well as in individuals with DM1. These results strongly support a role for CUGBP1 up-regulation in DM1 pathogenesis.

INTRODUCTION

Myotonic dystrophy is the second most common cause of muscular dystrophy and the most common cause of adult-onset muscular dystrophy (1). The predominant form of the disease in the USA, DM type 1 (DM1), is caused by a CTG expansion in the 3'-untranslated region (UTR) of the DMPK gene. Unaffected individuals commonly have up to 35 CTG repeats at this locus, whereas those affected by the disease have from 50 to several thousand repeats, the latter experiencing a more severe, congenital form of the disease. A second form of myotonic dystrophy is caused by a CCTG expansion in the first intron of ZNF9 (2). Symptoms in both DM1 and DM2 are multi-systemic and include skeletal muscle wasting, cardiac conduction defects, myotonia, cataracts and insulin resistance. The disease can also affect the endocrine system, the central nervous system and smooth muscle (1).

Cardiac manifestations occur in more than 80% of individuals with DM1 and can include dilated cardiomyopathy, prolongation of the PR interval, various degrees of atioventricular block and widening of the QRS complex (3–9). Conduction abnormalities often progress to life-threatening arrhythmias such that sudden death due to cardiac causes occurs in up to 30% of individuals with DM1 (5,9). In two longitudinal studies that followed more than 700 DM1 patients over a period of at least 5 years, cardiovascular disease comprised...
the second most common cause of mortality (5, 6). In addition to arrhythmias, cardiac manifestations also include non-ischaemic cardiomyopathy and specifically left ventricular systolic dysfunction.

In DM1, the expanded allele is transcribed to produce toxic RNA containing expanded CUG repeats (DMPK-CUGn RNA) that accumulates in nuclear foci and causes disease by at least two mechanisms (10). First, expanded CUG repeats fold into an imperfect double stranded hairpin structure that binds members of the muscleblind-like (MBNL) family of RNA-binding proteins resulting in their sequestration and depletion from the nucleus (11, 12). Second, DMPK-CUGn RNA activates protein kinase C (PKC) resulting in phosphorylation, stabilization and ultimately up-regulation of CUG-binding protein 1 (CUGBP1). This is the proposed mechanism for the observed 2–4-fold increase in steady state levels of CUGBP1 in DM1 heart and skeletal muscle tissues and in DM1 myoblast cultures (13–15). The loss of MBNL and gain of CUGBP1 activities have been proposed to contribute to DM1 pathogenesis. Both MBNL and CUGBP1 are RNA-binding proteins that regulate postnatal splicing transitions during striated muscle development (12, 16). The effect of the DMPK-CUGn RNA is to reverse the gain of MBNL1 and loss of CUGBP1 activities during normal postnatal developmental resulting in expression of embryonic splicing patterns for a number of genes resulting in disease symptoms (10).

We have previously generated a mouse model for DM1 using a Cre-LoxP approach to induce heart-specific expression of RNA containing DMPK exon 15 and 960 interrupted CUG repeats in adult animals (17). Expression of DMPK-CUG960 RNA is induced in adult animals using a heart-specific and tamoxifen-inducible form of Cre (MerCreMer or MCM) (18). Bitransgenic EpA960/MCM mice induced to express DMPK-CUG960 RNA exhibit systolic and diastolic dysfunction, prolongation of PR intervals, widening of the QRS complex and arrhythmias. These animals also reproduce multiple molecular features of DM1 including misregulated alternative splicing, RNA foci formation and co-localization of MBNL1 with RNA foci as well as up-regulation of CUGBP1 2–4-fold above endogenous levels (17).

Heart-specific overexpression of CUGBP1 results in lethality

The TRECUGBP1 transgene contains an N-terminal Flag-tagged version of the LYLQ isoform of human CUGBP1 located downstream of a tetracycline-responsive element (TRE) and minimal CMV promoter (Fig. 1A). Mice from TRECUGBP1 founder lines were mated with mice expressing a codon-optimized reverse tetracycline trans-activator (rtTA) specifically in heart driven by an α-MHC promoter (MHC-rtTA) (19). MHC-rtTA/TRECUGBP1 bitransgenic mice from two TRECUGBP1 founder lines were induced to express CUGBP1 using doxycycline delivered in food. Bitransgenic mice derived from the 3413 TRECUGBP1 line exhibited high penetrance of a phenotype of decreased movement and poor grooming by 7 days after dox administration and death within 14 days, while bitransgenic mice derived from the 3481 TRECUGBP1 founder line appeared overtly normal (data not shown). CUGBP1 overexpression was determined in bitransgenic mice derived from both lines 2, 4 and 7 days after initiation of doxycycline administration using anti-Flag antibodies. Bitransgenic mice from the 3413 founder line showed maximal transgene levels before developing lethargy at 7 days, whereas expression in mice derived from the 3481 founder line was lower (Fig. 1B). For both lines, Flag-tagged CUGBP1 is expressed only in bitransgenic mice given food containing doxycycline, whereas bitransgenic mice receiving standard food and MHC-rtTA mice administered doxycycline do not show detectable transgene expression (Fig. 1B and data not shown). Two-fold dilutions of heart extracts from individual mice from induced 3413 bitransgenic animals were probed with α-CUGBP1 antibody to estimate fold overexpression compared with endogenous CUGBP1 levels. CUGBP1 was induced to greater than 4-fold above endogenous levels in MHC-rtTA/TRECUGBP1 mice given doxycycline compared with control mice (Fig. 1C). This is consistent with an independent assessment of a 5–8-fold induction in bitransgenic animals from this line that was published previously (16). We conclude that 4-fold or higher cardiac-specific overexpression of TRECUGBP1 is sufficient to induce lethality. Some variability in transgene expression using this MHC-rtTA line was observed in our studies and has been previously noted (19) All other studies reported here were performed using mice from the 3413 line.
Acute overexpression of CUGBP1 leads to dilated cardiomyopathy

Hearts from bitransgenic animals given doxycycline were visibly larger than those of the control groups (Fig. 2A and B, Table 1). The heart weight to body weight ratio at sacrifice was significantly increased in induced bitransgenic MHC-rtTA/TRECUGBP1 mice compared with control animals (MHC-rtTA/TRECUGBP1 not treated with dox or MHC-rtTA treated with dox) (0.70 ± 0.03% versus 0.50 ± 0.01%, P < 0.001; Fig. 2B, Table 1). Histological findings in bitransgenic mice induced for 8 days included cardiomyopathy with widespread degeneration, necrosis and loss of myocardial fibers (Fig. 2C). Degenerating myocardial fibers exhibited a loss of granularity and striations with cytoplasmic vacuolization and condensation. No abnormalities were seen in bitransgenic animals that did not receive doxycycline, although MHC-rtTA mice given doxycycline showed a mild and scattered degenerative change. None of the control mice exhibited either overt illness or cardiac functional abnormalities (see below).

A side by side comparison of histology from mice induced to overexpress CUGBP1 (Fig. 2) and previously described bitransgenic EpA960/MCM mice induced to express DMPK-CUG960 RNA [published in Wang et al. (17)] revealed strong similarities including condensed darkly stained segments of fibers, moth-eaten appearance of other fibers, sarcoplasmic vacuoles, fiber atrophy and fiber loss. Similar histological changes are observed in heart tissue obtained from individuals with DM (see Discussion). We conclude that heart-specific, inducible CUGBP1 overexpression is sufficient to reproduce histopathological abnormalities seen in mice induced to express DMPK-CUG960 RNA as well as in individuals with DM1. Similarities between animals expressing exogenous CUGBP1 and DMPK-CUG960 RNA were also observed with regard to ECG and functional abnormalities (see below).

Induced overexpression of CUGBP1 reproduces disrupted alternative splicing changes seen in DM1

Disrupted alternative splicing is a characteristic molecular feature of DM1 (20,21). Specifically, alterations in developmentally regulated alternative splicing patterns are observed such that embryonic splicing patterns are observed in adult tissues. We recently demonstrated that a large number of alternative splicing transitions occur during

Figure 1. Generation of MHC-rtTA/TRECUGBP1 bitransgenic mice that inducibly overexpress human CUGBP1 in heart. (A) The TRECUGBP1 construct, shown to scale, expresses the human LYLQ isoform of CUGBP1 containing an N-terminal Flag tag driven by a TRE and a CMV minimal promoter. (B) Anti-Flag western blot using heart protein extracts showing a time course following transgene induction. MHC-rtTA/TRECUGBP1 bitransgenic animals from two TRECUGBP1 founder lines (3413 and 3481) sacrificed at 2, 4 and 7 days after 2 g doxycycline/kg food was initiated. (C) Quantification of CUGBP1 expression in MHC-rtTA/TRECUGBP1 animals from the 3413 line. Western blot analysis using anti-CUGBP1 antibodies of 12.5, 25 and 50 μg of protein extracts from hearts of induced (8 days) and uninduced MHC-rtTA/TRECUGBP1 bitransgenic animals, and MHC-rtTA animals were given doxycycline. Bitransgenic animals receiving doxycycline express total CUGBP1 levels four fold or higher compared with control mice.
normal mouse heart development and that more than half of these are responsive to changes in CUGBP1 and/or MBNL1 expression (16). Specifically, we demonstrated that 13 developmentally regulated splicing events were responsive to overexpression of CUGBP1 in MHC-rtTA/TRECUGBP1 mice but not the loss of MBNL1. CUGBP1 protein decreases 10-fold during postnatal heart development and all CUGBP1-responsive events revert to the embryonic pattern upon induction of CUGBP1 in adult heart (16).

We wanted to determine whether the splicing events that were previously shown to be responsive to CUGBP1 but not MBNL1 were affected by DMPK-CUGn RNA expression in either induced EpA960/MCM mice or individuals with DM1. We used RT–PCR (Fig. 3A) to test four CUGBP1-responsive, MBNL1-non-responsive splicing events in induced EpA960/MCM mice (C10orf97, Mfn2, Capzb and Ppfibp1). Two events that are responsive to MBNL1 were also tested (cTNT and Ablim1). Aberrant regulation of
cTNT exon 5 in adult DM1 heart was previously shown to be indicative of splicing changes associated with the disease (22). All six events reverted to embryonic splicing patterns in induced EpA960/MCM mice but not in control mice that did not express DMPK-CUG RNA (Fig. 3B and Supplementary Material, Fig. S1). The response of the MBNL1-responsive splicing events reflects the previously described sequestration of MBNL1 protein. The response of the CUGBP1-responsive, MBNL1-non-responsive splicing events shown in Figure 3B demonstrates a functional consequence of the CUGBP1 up-regulation that is observed in these mice (17). Of the three induced EpA960/MCM + tam mice tested, one showed a mild effect on all splicing events likely reflecting poor induction of DMPK-CUG960 RNA.

We also tested whether the same CUGBP1-responsive, MBNL1-non-responsive splicing events were altered in heart tissue from individuals with DM1 compared with tissues from unaffected individuals and individuals with non-DM causes of dilated cardiomyopathy. As presented in Figure 3C, these splicing events are disrupted in heart tissue from at least some individuals with DM1 compared to unaffected controls. These transitions are specific to DM1 as they are not observed in non-DM forms of dilated cardiomyopathy (Fig. 3C). Comparison of alternative splicing in fetal and adult heart tissues demonstrates that all six alternative splicing events are regulated during human heart development (Fig. 3C) as previously demonstrated in mouse (16). The splicing patterns of the four CUGBP1-mediated events revert to the embryonic pattern in DM1 heart tissues, consistent with increased CUGBP1 activity (16). Because these splicing events are not responsive to MBNL1 depletion in mice, and the splicing pattern is consistent with increased CUGBP1 activity, we conclude that the elevated expression of CUGBP1 in heart tissue of EpA960/MCM mice and individuals with DM1 is driving the embryonic splicing pattern of these events. Thus, inducible, heart-specific overexpression of CUGBP1 is sufficient to reproduce disrupted alternative splicing seen in EpA960/MCM mice and DM1 patient tissues.

### Cardiac-specific overexpression of CUGBP1 causes dilated cardiomyopathy

To determine the effects of CUGBP1 overexpression on heart structure and function, echocardiography was performed on induced MHC-rtTA/TRECUGBP1 bitransgenic and control mice. Data from MHC-rtTA/TRECUGBP1 (+dox) mice and MHC-rtTA (+dox) mice were grouped as controls for statistical tests since neither group express exogenous CUGBP1 (Fig. 4B–D). Echocardiography was performed on induced and control animals 1 day prior to doxycycline administration and then at 3, 5–6 and 7–8 days following initiation of doxycycline administration. Representative echocardiograms from the same two animals are shown the day before and 8 days after initiation of doxycycline administration (Fig. 4A).

Transgenic mice overexpressing CUGBP1 but not control mice exhibited dilated cardiomyopathy based on the findings...
of left ventricular systolic dysfunction with accompanying left ventricular dilatation (23). Left ventricular ejection fraction, a measure of systolic function, was significantly decreased in animals overexpressing CUGBP1 compared with control mice at all time points after doxycycline administration (Fig. 4B and Table 1). The reduction in ejection fraction was most striking 7–8 days after starting doxycycline (28.0 ± 3.7% in dox-treated MHC-rtTA/TRECUGBP1 mice versus 67.2 ± 1.2% in the controls, respectively, P < 0.001).

Left ventricular dilation was evidenced by an increase in both left ventricular internal diameter in diastole (LVIDd) (Fig. 4C and Table 1) and LVID in systole (LVIDs) (Fig. 4D and Table 1). The increased LVIDs was also indicative of systolic dysfunction in dox-treateed MHC-rtTA/TRECUGBP1 mice. Specifically, both LVIDd and LVIDs were significantly increased in the experimental group compared with controls at all time points after initiation of doxycycline administration. For example, by days 7–8, LVIDd 4.12 ± 0.08 mm versus 3.62 ± 0.07 mm; LVIDs 3.65 ± 0.17 mm versus 2.39 ± 0.08 mm (P < 0.001 for all measurements).

Another indicator of systolic dysfunction was decreased left ventricular posterior wall thickness during systole (LVPWs). Specifically, LVPWs was significantly (P < 0.05) decreased in experimental animals compared with controls after doxycycline initiation (0.94 ± 0.03 mm versus 1.06 ± 0.04 mm at days 7–8, respectively, Table 1).

In summary, left ventricular dilated cardiomyopathy, a combination of systolic dysfunction and ventricular dilation, is seen in induced bitransgenic MHC-rtTA/TRECUGBP1 mice. A similar decreased left ventricular systolic function and dilated cardiomyopathy is observed in both the EpA960/MCM DM1 mouse model (17) and in DM1 patients (4,7,24). We conclude that overexpression of CUGBP1 in cardiomyocytes results in dysfunctional contraction and is sufficient to reproduce echocardiographic abnormalities seen in an inducible, heart-specific mouse model expressing DMPK-CUG RNA and in individuals with DM1.

Mice induced to overexpress CUGBP1 reproduce conduction abnormalities seen in DM1 patients

To analyze electrocardiographic activity in unanesthetized mice, we use ECG telemetry. Representative electrocardiograms are shown for MHC-rtTA/TRECUGBP1 and MHC-rtTA mice before doxycycline administration and 7 or 8 days after doxycycline administration, respectively (Fig. 5A). MHC-rtTA/TRECUGBP1 but not MHC-rtTA mice exhibited a significant doxycycline-induced increase in the PR interval duration (8.69 ± 1.45 ms versus 4.60 ± 0.78 ms, respectively, P < 0.05) (Table 1, Fig. 5B). Similarly, an increase in the QRS complex width was observed in MHC-rtTA/TRECUGBP1 mice when compared with MHC-rtTA controls following administration of doxycycline (5.63 ± 1.42 ms versus 1.79 ± 0.34 ms, respectively, P < 0.05) (Fig. 5B and Table 1). Similar to the structural and functional changes induced by CUGBP1 overexpression described above, both increased PR interval and QRS complex duration has been observed in EpA960/MCM mice induced to express DMPK-CUG960 RNA (17) and in DM1 patients (17,25). We conclude that overexpression of CUGBP1 in adult mouse heart.
reproduces both increases in PR interval and QRS complexes as observed in DM1 patients and in the EpA960/MCM heart DM1 mouse model.

DISCUSSION

In DM1, DMPK-CUGn RNA has at least two pathogenic effects: depletion of MBNL proteins by sequestration and up-regulation of CUGBP1 via PKC-mediated activation. We have previously shown in a mouse model of DM1 (EpA960/MCM) that heart-specific expression of DMPK-CUG960 RNA in adult EpA960/MCM mice reproduces functional and electrophysiological abnormalities observed in DM1. These mice also reproduced molecular features observed in DM1 heart tissue, including 2–4-fold up-regulation of CUGBP1 (17). Here we tested the hypothesis that up-regulation of CUGBP1 is responsible for at least some of the pathological features observed in mice expressing heart-specific DMPK-CUG960 RNA.

There are several pieces of evidence supporting a role for up-regulated CUGBP1 expression in DM1 pathogenesis. CUGBP1 protein levels are elevated in DM1 heart and skeletal muscle tissues and cultured cells (13–15). Constitutive transgenic overexpression of CUGBP1 primarily in skeletal muscle as well as low expression in heart beginning around embryonic day 16 using the mouse creatine kinase promoter resulted in neonatal lethality due likely to respiratory failure. These mice exhibit dystrophic muscle and DM1-like splicing changes (26). An independent transgenic analysis of CUGBP1 overexpression primarily in skeletal muscle demonstrated developmental delay of skeletal muscle maturation (27). Furthermore, CUGBP1 was shown to enhance the skeletal muscle phenotype of a Drosophila model of DM1 (28).

Here we show that inducible, cardiac-specific overexpression of CUGBP1 in adult animals results in premature lethality, cardiomyocyte pathology, alternative splicing changes and functional (both echocardiographic and electrocardiographic) findings consistent with cardiac features observed in individuals with DM1. The pathological features of CUGBP1-overexpressing mice also strongly resembled our previously published EpA960/MCM mouse model of DM1 (17). Induced bitransgenic MHC-rtTA/TRECUGBP1 mice overexpress CUGBP1 protein four to eight times above endogenous levels compared with uninduced bitransgenic animals and MHC-rtTA mice given doxycycline. This level of CUGBP1 expression is somewhat higher than what was observed in hearts from induced EpA960/MCM animals as well as in DM1 heart tissue (2–4-fold) (17) and could explain the severe phenotype of lethality within 2 weeks of CUGBP1 induction. Comparisons of induced MHC-rtTA/TRECUGBP1 mice that overexpress CUGBP1 and induced EpA960/MCM mice that express DMPK-CUG960 RNA revealed similarities in histological changes, alternative splicing changes and disrupted cardiac function that are also observed in individuals with DM1. For example, studies comparing DM1 patients to age-matched controls have shown decreased ejection fraction as is seen in induced EpA960/MCM mice and induced bitransgenic MHC-rtTA/TRECUGBP1 mice (4,24,25). Light microscopic analysis of DM1 patient heart tissues typically reveals non-specific myopathic changes (25,29–31). Some common features, however, have been shown in the literature. Many...
of these features are reproduced in induced MHC-rtTA/TRECUGBP1 mouse cardiac tissues. Focal loss of myofilaments, as seen in these mice, has previously been noted in DM1 patients (29,31). Cytoplasmic vacuolization has also been noted in the hearts of both induced MHC-rtTA/TRECUGBP1 mice and DM1 patients (29,30). The rapid onset of disease and lethality in induced MHC-rtTA/TRECUGBP1 mice may explain why other characteristic histopathological findings of DM1 patient hearts—myocyte hypertrophy, fatty infiltration and interstitial fibrosis—were not seen (25).

CUGBP1 expression in heart is normally developmentally regulated in mouse such that protein levels decrease 10-fold after postnatal day 6. Interestingly, CUGBP1 mRNA levels do not change indicative of regulation at the level of translation or post-translational protein stability (16). CUGBP1 protein stability is increased by phosphorylation and the loss of CUGBP1 phosphorylation correlates with its postnatal down-regulation. The effect of DMPK-CUGn RNA is to induce CUGBP1 hyperphosphorylation through a PKC-dependent mechanism to enhance its stability and increase steady-state protein levels (32). Here we showed that elevation of CUGBP1 in the absence of DMPK-CUGn RNA expression or MBNL1 depletion is sufficient to reproduce several key functional and splicing abnormalities observed in the DM1 mouse model expressing DMPK-CUGn RNA in heart as well as observed in individuals with DM1.

These results demonstrate that MBNL1 depletion and CUGBP1 up-regulation have independent as well as overlapping downstream consequences that are relevant to disease pathogenesis. Such an understanding is important for full consideration of specific therapeutic options. For example, targeting either the MBNL1 or CUGBP1 pathway alone will not address all consequences of DMPK-CUGn RNA expression. Combinatorial therapeutic approaches are likely to have an additive benefit.

MATERIALS AND METHODS

Transgenic mice

An N-terminal Flag-tagged version of the LYLQ isoform of human CUGBP1 was expressed from a transgene containing (from 5' to 3') a TRE/minimal CMV promoter (33), a genomic fragment containing α-MHC untranslated exons 2 and 3 with intron 2, the CUGBP1 open-reading frame and the bovine growth hormone polyadenylation site and 3' flanking genomic segment (obtained from the pBluescript vector, Stratagene, La Jolla, CA, USA) for proper mRNA 3' end formation. The LYLQ isoform was used because it predominates in human striated muscle (unpublished data) and includes 12 nucleotides encoding LYLQ due to use of an alternative 3' splice site.
Because 480 of 486 residues are identical (98.8%) between mouse and human CUGBP1 proteins, the possibility that the exogenous protein will initiate an immune response is low. Transgenic mice were generated by standard techniques and maintained on an FVB background. For genotyping, genomic DNA was extracted from mouse tails using DirectPCR lysis reagent (Viagen Biotech, Los Angeles, CA, USA) and analyzed by multiplex PCR using a β-actin internal control. The following primers were used: TRECUGBPf (TTGGCTGTAAAAATTGGGCTCCT) and actin1 (GATGTGCTCCAGGCTAAAGTT), actin2 (AGAAACGGAATGTTGTGGAGT). Of three transgenic lines shown to express exogenous CUGBP1 upon induction, the line expressing the highest amount (4–8-fold) of CUGBP1 (line 3413) was used. The line expressing the next highest level of CUGBP1 (3481) produced approximately two times above endogenous levels of CUGBP1.

MHC-rtTA transgenic mice (FVB/N-Tg(Myh6-rtTA)1Jam) expressing a codon-optimized rtTA variant specifically in heart were commercially obtained (MMRRC, UNC, Chapel Hill, NC, USA) (19). All mice reported were F1 progeny of TRECUGBP1 × MHC-rtTA matings and were therefore hemizygous for one or both transgenes. Bitransgenic, transgenic and wild-type mice used for all experiments were littermates whenever possible. Doxycycline was given in the food (2 g doxycycline/kg food, Bio-Serv, Frenchtown, NJ, USA) to animals that were 2–6 months of age. Body weights were followed daily. For histological analysis, whole hearts were immediately placed in 10% formalin overnight before undergoing processing for histology, sectioning and staining. Heart sections were stained using hematoxylin and eosin and Masson's trichrome. All experiments involving mice were conducted in accordance with the NIH Guide for the Use and Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Immunoblots
Protein was extracted with a dounce homogenizer in 10 mM HEPES (pH 7.5), 0.32 M sucrose, 1% SDS, 5 μM MG132 and 5 mM EDTA with protease inhibitors. Except where noted, 50–70 μg of total protein per lane was used for western blotting. The membrane was incubated with monoclonal anti-FLAG antibody (M2; Sigma-Aldrich, St Louis, MO, USA) or anti-CUGBP1 (3B1; Upstate, Lake Placid, NY, USA) conjugated with HRP (34).

RT–PCR splicing analysis
RT–PCR assays were performed on total RNA extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was oligo-dT primed and PCR was performed as described previously (16). The percentage of exon inclusion was calculated using Kodak (Rochester, NY, USA) Gel Logic 2200 and Molecular Imaging Software as: [exon inclusion band/(exon inclusion band + exon exclusion band)] × 100]. Primer sequences for the mouse were described previously (16) and for human are as follows: C10orf97, Mfn2, Capzb, Ppifbp1, cTNT and Ablim1. DM1 patient and normal human tissue samples were obtained from C. Thornton (University of Rochester, Rochester, NY, USA) and from the NRDI tissue cooperative. The following primers were used for RT-PCR: Hum-Capzbf (GGATATCGTATGGCCTAG), Hum-Capzbr (GGATATCGTATGGCCTAG), Hum-Mfn2f (GTCCATGATGCTACCCGCT), Hum-Mfn2r (TCTTGGCACTGACAAAGTGC), Hum-Ablim1f (GACCTGGTGAGAGGAGGAG), Hum-Ablim1r (GACCTGGTGAGAGGAGGAG). All data are expressed as mean ± SEM. Statistical significance was determined using the two-tailed, two-sample Student’s t-test. The F-test for unequal variances was performed to determine whether Student’s t-test for equal or unequal variances was to be performed. A P-value less than 0.05 was considered significant.

**SUPPLEMENTARY MATERIAL**
Supplementary Material is available at HMG online.
REFERENCES


ACKNOWLEDGEMENTS
We thank Donnie Bundman, Anthony Vu, Joey Cienfuegos and Tomohiko Ai for technical assistance. We also thank members of the Cooper and Wehrens laboratories for helpful discussions. We also thank Charles Thornton (Univ. Rochester) and the National Disease Research Interchange for patient tissue samples.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by NIH predoctoral NSRA fellowship F30NS61358-01 (M.V.K.), the Baylor Research Advocates for Student Scientists (BRASS) Program (M.V.K.), NIH grants R01AR45653 and R01GM076493 (T.A.C.), the Muscular Dystrophy Association (T.A.C.), NIH grants R01089598 and R01091947 (X.H.W.), the Muscular Dystrophy Association (X.H.W.) and the W.M. Keck Foundation Distinguished Young Scholars program (X.H.W.).