The Mef2 Transcription Network Is Disrupted in Myotonic Dystrophy Heart Tissue, Dramatically Altering miRNA and mRNA Expression

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SUMMARY

Cardiac dysfunction is the second leading cause of death in myotonic dystrophy type 1 (DM1), primarily because of arrhythmias and cardiac conduction defects. A screen of more than 500 microRNAs (miRNAs) in a DM1 mouse model identified 54 miRNAs that were differentially expressed in heart. More than 80% exhibited downregulation toward the embryonic expression pattern and showed a DM1-specific response. A total of 20 of 22 miRNAs tested were also significantly downregulated in human DM1 heart tissue. We demonstrate that many of these miRNAs are direct MEF2 transcriptional targets, including miRNAs for which depletion is associated with arrhythmias or fibrosis. MEF2 protein is significantly reduced in both DM1 and mouse model heart samples, and exogenous MEF2C restores normal levels of MEF2 target miRNAs and mRNAs in a DM1 cardiac cell culture model. We conclude that loss of MEF2 in DM1 heart causes pathogenic features through aberrant expression of both miRNA and mRNA targets.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is an autosomal-dominant disease caused by an expanded CTG repeat in the last exon of the dystrophia myotonica-protein kinase (DMPK) gene. Pathogenesis is caused primarily by the mRNA containing expanded CUG repeats (CUGexp RNA) that is expressed from the mutated allele (Wheeler and Thornton, 2007). DMPK is expressed in multiple tissues that are subsequently affected in the disease, but the primary causes of mortality are muscle wasting (60%) and sudden cardiac death (25%–30%) (Groh et al., 2008; Heatwole et al., 2012; Phillips and Harper, 1997; Salehi et al., 2007). More than 80% of individuals affected with DM1 have cardiac conduction defects and arrhythmias, and a lower percentage are affected by interstitial fibrosis and dilated cardiomyopathy (Groh et al., 2008; Lazarus et al., 2002; Nazarian et al., 2010; Pelargonio et al., 2002; Phillips and Harper, 1997; Sovari et al., 2007). Although several molecular mechanisms of DM1 pathogenesis have been defined (Sicot et al., 2011; Udd and Krahe, 2012), the specific mechanisms causing electrophysiological, fibrotic, and contractility abnormalities in DM1 heart tissue are unknown.

The best-characterized effects of CUGexp RNA are disrupted functions of the RNA binding proteins muscleblind-like 1 (MBNL1) and CUGBP and Elav-like family member 1 (CELF1), which regulate multiple RNA-processing events including alternative splicing, translation, mRNA stability, and mRNA intracellular localization (Lee and Cooper, 2009; Timchenko, 2013). Celf1 is downregulated during mouse postnatal heart and skeletal muscle development while Mbnl1 activity is upregulated, driving their target alternative splicing events to the adult pattern (Kalsotra et al., 2008; Lin et al., 2006). Celf1 downregulation is posttranscriptionally mediated by microRNA (miRNA)-repressed translation and protein destabilization by dephosphorylation (Kalsotra et al., 2008, 2010; Kuyumcu-Martinez et al., 2007). CUGexp RNA reverses normal postnatal regulation of MBNL1 and CELF1 by sequestration of MBNL1, which binds with high affinity to the CUG repeats, and stabilization of CELF1 by PKC-activated phosphorylation, resulting in a 2- to 4-fold increase in heart and skeletal muscle (Kuyumcu-Martinez et al., 2007; Savkur et al., 2001; Timchenko et al., 2001; Wang et al., 2007). In addition to disrupted alternative splicing, molecular defects of CUGexp RNA toxicity involve repeat-associated...
non-ATG (RAN) translation (Zu et al., 2011), abnormal DNA methylation (López Castel et al., 2011), bidirectional transcription (Moseley et al., 2006), and miRNA dysregulation (Fernández-Costa et al., 2013; Perbellini et al., 2011; Rau et al., 2011).

We previously demonstrated that postnatal downregulation of Celf1 and its paralog Celf2 in mouse heart results from a dramatic upregulation of miR-23a and miR-23b between postnatal day 2 (PN2) and PN21 (Kalsotra et al., 2008, 2010). Therefore, we wanted to determine whether altered miRNA expression in DM1 could be an additional mechanism of CELF1 upregulation. Using an established heart-specific and inducible DM1 mouse model, we found that postnatal upregulation of miR-23a and miR-23b is dramatically reversed upon induction of CUG<sup>exp</sup> RNA in adult heart. Furthermore, an analysis of >500 miRNAs identified 54 that are misregulated within 72 hr of CUG<sup>exp</sup> RNA induction, >80% of which represent reversal of postnatal upregulation. A total of 20 of 22 miRNAs affected in the DM1 mouse model were also downregulated in DM1 heart tissues. Pathway analysis of miRNAs and miRNAs misregulated in the DM1 mouse heart identified a loss of function of the Mef2 transcriptional network. Loss of MEF2A and MEF2C mRNA and protein expression was demonstrated in heart tissue from the DM1 mouse model and in individuals affected by DM1. In addition, 20 of 20 protein-coding genes that are demonstrated targets of MEF2 were downregulated in the DM1 mouse model. Misregulation of miRNA and mRNA MEF2 targets by CUG<sup>exp</sup> RNA was rescued by MEF2C. For several of the affected miRNAs, downregulation has previously been shown to produce arrhythmias or fibrotic changes. Our results demonstrate that the MEF2 transcription network is disrupted by CUG<sup>exp</sup> RNA, leading to altered expression of a large number of miRNA and mRNA targets with effects consistent with DM1 heart pathology.

**RESULTS**

**Disrupted Expression of Postnatally Regulated miRNAs in Adult DM1 Heart**

To determine whether CELF1 upregulation in DM1 heart tissue results from altered miRNA expression, we quantified miR-23a and miR-23b expression in heart tissue from a heart-specific DM1 mouse model (EpA960; MerCreMer [MCM]). These mice and 23a resulted from altered miRNA expression, we quantified To determine whether CELF1 upregulation in DM1 heart tissue resulted from altered miRNA expression, we quantified To determine whether CELF1 upregulation in DM1 heart tissue resulted from altered miRNA expression, we quantified To determine whether CELF1 upregulation in DM1 heart tissue resulted from altered miRNA expression, we quantified...
miRNAs. We found that ten out of ten primary miRNA transcripts examined showed decreased expression that paralleled the reduced expression of their mature miRNAs when assayed at 72 hr and 1 week after CUG<sup>exp</sup> RNA induction (Figure 2E). Moreover, we found that expression of pri-miR-1-1 and pri-miR-1-2 are significantly reduced in the hearts of DM1 patients relative to unaffected controls (Figure 2F). Overall, these data demonstrate that gain of Cel1f or loss of Mbn1 activity is not responsible for the altered miRNA expression in DM1. Our data are consistent with an upstream defect in transcription rather than a downstream RNA processing defect.

Large-Scale Shift in Gene Expression in DM1 Is Partly due to Loss of miRNAs and Inactivation of the Met2 Transcriptional Program

To assess if in addition to splicing and miRNA defects, CUG<sup>exp</sup> RNA also perturbed mRNA steady-state levels, we carried out a microarray study on heart RNA from wild-type E14 and adult mice as well as adult MCM control and DM1 mice. As anticipated, we noted a large number of genes to be developmentally regulated in wild-type hearts; however, within 72 hr of CUG<sup>exp</sup> RNA induction, many genes showed a coordinated adult-to-embryonic shift in mRNA expression (Figure 3A). Strikingly, 1 week of CUG<sup>exp</sup> RNA expression resulted in a pervasive shift in transcript levels of a large number of genes toward the embryonic pattern (Figure 3A).

Gene Ontology analysis using the Ingenuity Pathway Analysis (IPA) showed the mitochondrial pathway as the most significantly affected pathway (p = 5.36 × 10<sup>-6</sup> and threshold ratio = 0.259) (Figure S2). To identify the transcription factors and miRNAs that are potentially responsible for these gene expression changes and the ensuing phenotype in DM1 mice, we performed an upstream regulator analysis by IPA. This analysis examined the enrichment of known targets of each
transcriptional regulator present in our gene list to that in the database, resulting in an estimation of an overlap p value. Based on activation or suppression of target genes (for a transcriptional regulator) compared with observed changes in gene expression, an activation Z score was assigned. Z score > 2 illustrates activation and Z score < -2 illustrates inhibition of activity. Using this approach, we discovered the cardiac transcription factors Mef2a and Mef2c as most significantly inhibited (Mef2a Z score of -2.941, p = 1.70 × 10^{-10}; Mef2c Z score of -3.017, p = 1.20 × 10^{-13}) (Figure 3B; Table S2).

IPA also predicted inhibition of miRNA families miR-29, let-7, miR-1, and miR-34a, corroborating the reciprocal upregulation of their corresponding targets (Table S2). Finally, to understand how Mef2 and the predicted miRNAs interact with one another and their targets, we made an interaction network and overlaid it with the cardiac disease function from the function and disease tools of IPA. These analyses showed genes involved in cardiac arrhythmia, hypertrophy, and fibrosis as overrepresented in the network (Figure 3B). Importantly, these categories correlate strongly with the phenotypic changes observed in DM1 patients and mice, including prolonged PR intervals and QRS duration, decreased contractility, dilated cardiomyopathy, hypertrophy of cardiomyocytes, and proliferation of mitochondria (Wang et al., 2007).

Quantitative real-time RT-PCR assays confirmed significant downregulation (p < 0.05) of Mef2a and Mef2c mRNAs in our DM1 mouse model (Figure 4A, top) as well as in DM1 patient samples (Figure 4A, bottom). As a control, we assessed Gata4 transcript levels, which were unchanged in both the DM1 mouse model and DM1 heart tissues. MEF2A and MEF2C mRNAs were not affected in human heart failure samples (Figure S3D), indicating that reduced expression is not a general response to heart disease. We also found that the alternative exons in Mef2a (α and β exons) and Mef2c (γ exon) did not exhibit a significant difference in percent spliced in (PSI) values following 1 week of CUG<sup>exp</sup> RNA induction in DM1 mice. Each bar represents fold change in individual miRNA expression (mean ± SD). Data are normalized relative to U6 snRNA (n = 3). (D) Real-time PCR analysis monitoring PSI of two Mbnl1-regulated alternative splicing events, Mtmr3 exon 16 and Mfn2 exon 3 in MHCrtTA or TgCEL1F1 mice given dox or Mbnl1<sup>AE3/AE3</sup> mice. (E) miRNA expression in hearts of Mbnl1<sup>AE3/AE3</sup> relative to Mbnl1<sup>+/-</sup> mice showing fold change in individual miRNA expression (mean ± SD). Data are normalized relative to U6 snRNA (n = 3). (F) Reduced steady-state levels of pri-miR-1-1 and pri-miR-1-2 transcripts in human heart samples from DM1 patients relative to unaffected individuals (n = 3).

* p < 0.05.
CARDIOVASCULAR GENE FUNCTION CATEGORIES WITH \( p < 10^{-3} \)

such targets were chosen (see the Supplemental Information). Remarkably, 100% (20/20) of the Mef2 target genes examined were downregulated in DM1 mouse heart tissue (Figure 4C, left). As a control data set, we tested a target gene set for Gata4 (see the Supplemental Information), which is unaffected in DM1 heart tissue (Figure 4A). We observed no significant change in expression of Gata4 targets in DM1 mice (Figure 4C, right). These results indicate that the vast majority of experimentally supported Mef2 target genes expressed in heart are downregulated in DM1.

**Identification of Mef2-Regulated miRNAs in Cardiac Cells**

In addition to many muscle-specific genes, Mef2 directly activates transcription of bicistronic primary transcripts encoding \( \text{miR-1-2}^{*} / \text{miR-1-1}^{*} - \text{miR-133a-1} \) and \( \text{miR-1-1}^{*} / \text{miR-133a-2} \) (Liu et al., 2007), which we found to be downregulated in DM1 patient and mouse model heart tissues. However, it is not known what other miRNAs are regulated by Mef2 in heart. We searched 10 kb genomic regions spanning each of the 54 differentially expressed miRNAs in DM1 mice for Mef2 binding sites [CTA(A/T)_{n}TAG] and found 65 putative sites in 34 different miRNA genes.

To determine the fraction of miRNA genes physically bound by Mef2 proteins in heart, we performed ChIP assays on wild-type adult mouse hearts using a pan-Mef2 antibody. Nonimmune immunoglobulin Gs (IgGs) and RNA Pol II antibodies served as negative and positive controls, respectively. The genomic regions harboring the ChIP-ed Mef2 consensus sites were amplified using specific primer sets. PCR analyses of the precipitated chromatin showed strong Mef2 binding along the genomic regions of previously characterized Mef2 targets (Smyd1, Fgc1a, Myom1, and Ctnna3), whereas no significant binding was detected to an intergenic negative control region (Figure S4A).

Using the ChIP assay, we confirmed that 20 of 65 sites in 15 different pri-miRNAs were occupied by Mef2 (Figure S4B). The let-7d upstream region does not contain a Mef2 site and showed no binding, whereas a previously characterized pri-miR-1-2 binding site showed positive binding, as expected. RNA Pol II showed preferential association with all miRNA and mRNA genomic regions tested, confirming these are actively transcribed in wild-type mouse hearts (Figure S4B).

To determine whether expression of the miRNAs downregulated in DM1 heart require Mef2, we used small interfering RNAs (siRNAs) to knock down Mef2a and Mef2c genes individually or in combination in mouse atrial cardiac HL-1 cells. We consistently achieved over 80% knockdown efficiency for both Mef2a and Mef2c from their endogenous levels (Figure 5A). We tested expression of three known Mef2 target genes (Myocd, Myom1, and Ctnna3) in Mef2 knockdown cultures and observed an expected reduction in their steady-state levels compared to the control knockdowns (Figure 5A). Importantly, both individual and combined knockdowns resulted in a significantly lower expression (\( p < 0.05 \)) of 10 out of 15 pri-miR transcripts that showed Mef2 binding in the ChIP assay (Figure 5B; Figure S4B). A total of 4 out of 15 were not expressed in HL-1 cells, whereas pri-miR-30a was expressed but was unaffected by the knockdowns (Figure 5B).

These results indicate that in addition to pri-miR-1-1/133 clusters, Mef2 proteins drive expression of

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**Figure 3. The DM1 Heart Mouse Model Shows a Large-Scale Shift in Gene Expression that Identifies a Disrupted Mef2 Network**

(A) Gene expression profiling in mouse heart development and adult DM1 mice shows a developmental reversion in mRNA expression. Heatmap representation of transcripts overexpressed (yellow) and underexpressed (blue) in hearts of wild-type adult mice, wild-type embryonic day 14 (E14), and DM1 mice induced to express CUG\(^{agg} \) RNA for 72 hr and 1 week, when compared to MCM controls (\( p < 0.01 \); fold change > 1.5). Rows, transcripts (values centered on MCM group); columns, profiled samples. (B) Ingenuity Pathway Analysis identified Mef2 as a key regulator of both miRNA and mRNA with altered expression in heart tissue expressing CUG\(^{agg} \) RNA. Cardiovascular gene function categories with \( p < 1 \times 10^{-5} \) are highlighted in the figure.
several other pri-miRNAs in cardiac cells. We next used a pan Mef2 antibody to perform ChIP analysis of DM1 mouse heart tissue. Consistent with reduced Mef2a and Mef2c levels we noted their association with the response elements was decreased on both miRNA and mRNA targets in DM1 mouse heart tissues in comparison to the MCM controls (Figure 5C). Collectively, these data demonstrate that loss of Mef2 expression in DM1 is likely to have a significant impact on expression of downstream targets directly due to their reduced occupancy on the target genes.

**Misregulation of miRNAs and mRNAs in a DM1 Cardiac Cell Model Is Rescued by Exogenous Mef2c**

To test whether re-expression of Mef2 could rescue the loss of expression of select miRNA and mRNA targets in DM1, we infected HL-1 cells with control or tet-inducible Mef2c lentivirus that coexpresses an rtTA transactivator (Figure 6A) (Lee et al., 2012). After a 12 hr induction of Mef2c expression with doxycycline, cells were transfected with pBi-tet-DT0-GFP or -DT960-GFP plasmids. These plasmids express GFP and the DMPK 3′ UTR with or without 960 CUG repeats through a tet-inducible bidirectional promoter (Figure 6A) (Lee et al., 2012). Thirty-six hours later, cells were either fixed for combined fluorescence in situ hybridization/immunofluorescence analysis or lysed to extract total RNA. As shown previously (Lee et al., 2012), we found that most transfected cells that expressed GFP also formed CUG-repeat-containing RNA foci (Figure 6B). GFP expression or foci formation was not observed in the absence of doxycycline (Figures 6B and 6C), and no significant differences in DMPK mRNA levels were noted upon exogenous Mef2c expression (Figure 6C).

Similar to the DM1 mouse model and the human patient samples, transient transfection of HL-1 cells with the DT960 plasmid led to a significant decrease in Mef2a and Mef2c transcript levels when compared to the DT0 plasmid (Figure 6D). Forced expression of exogenous Mef2c in the DM1 cardiac cell model not only increased the Mef2a and Mef2c transcript levels (Figure 6D) but also rescued the expression of its mRNA and miRNA targets (Figures 6E and 6F). These results thus provide direct evidence that misregulation of the cardiac Mef2 regulatory network plays a fundamental role in the pathological response to CUG<sup>exp</sup> RNA in DM1 hearts.

**DISCUSSION**

We demonstrated a hierarchical relationship between expression of CUG<sup>exp</sup> RNA and loss of Mef2 activity using two independent experimental systems (an inducible heart-specific DM1 mouse model and a DM1 cardiac cell culture model) and validated the results in DM1 heart tissue. CUG<sup>exp</sup> RNA leads to an overall decrease in MEF2 expression and decreased expression of MEF2 miRNAs and mRNA targets resulting in global reprogramming of the cardiac transcriptome. Our results identify several miRNA families that are deregulated in DM1 heart tissues. This is predicted to have a cascade effect, as individual miRNAs can target multiple mRNAs (Bartel, 2009) and therefore modulate DM1 phenotype by regulating functionally related networks. For instance, miR-1 is known to regulate gap junction proteins and cardiac channels, including Gja1, Cacna1c, and Kcnq2, and a greater than 50% reduction in its expression may directly contribute to the conduction defects seen in DM1 (Rau et al., 2011; Zhao et al., 2007). This is consistent with a previous report where genetic loss of one of the two miR-1 family members resulted in a range of cardiac abnormalities, including postnatal electrophysiological defects with a spectrum of cardiac arrhythmias (Zhao et al., 2007).

Interstitial fibrosis is another important feature of DM1 heart tissue (Nazarian et al., 2010). Our study identified dysregulation of a network of four miRNA families that may be directly responsible for this phenotype. CUG<sup>exp</sup> RNA expression leads to an upregulation of miR-21 and downregulation of miR-29, miR-30, and miR-133 family members. miR-21 is known to repress the transcription of the cardiac arrhythmias (Zhao et al., 2007).

Intercalated disc disorganization is another important feature of DM1 heart tissue (Nazarian et al., 2010). Our study identified dysregulation of a network of four miRNA families that may be directly responsible for this phenotype. CUG<sup>exp</sup> RNA expression leads to an upregulation of miR-21 and downregulation of miR-29, miR-30, and miR-133 family members. miR-21 is known to repress the transcription of the cardiac arrhythmias (Zhao et al., 2007).
Our microarray study, and miR-30 and miR-133 repress expression of the connective tissue growth factor (Duisters et al., 2009), a positive regulator of fibrosis (4.8-fold upregulated in our study). Thus, the results from this study show tight reciprocal relationships between gain and loss of these four miRNAs and their target genes that support the critical role of this core network in DM1 cardiac fibrosis.

One of the clear downstream implications of miRNA dysfunction in DM1 is that reduced miR-23a/b levels lead to increased expression of its target, CELF1 protein. The miR-23a/b family regulates posttranscriptional loss of CELF1 protein during mouse embryonic heart development (Kalsotra et al., 2008; Lin et al., 2006). Reduced expression of miR-23a/b family members results in increased CELF1 protein. The miR-23a/b family is part of the miR-23a/b cluster, which is one of the miRNA clusters known to be involved in heart development (Ieda et al., 2010; Qian et al., 2012). Reduced levels of miR-23a and miR-23b in DM1 heart tissue, therefore, is expected to result in an overall increase in CELF1 protein levels, thus contributing to misregulation of CELF1 splicing targets. Together, these data indicate that dysregulation of specific miRNAs are likely to contribute to specific cardiac phenotypes observed in DM1.

MBNL1 and CELF1 are RNA binding proteins that are required for alternative splicing regulation during normal skeletal muscle and heart development (Kalsotra et al., 2008; Lin et al., 2006). Disruption of their functions by CUG expansion RNA results in missplicing of their pre-mRNA targets such that adult tissues express embryonic splice forms. It was recently described that reduced expression of miR-1 in DM1 patients is due in part to misprocessing of pre-miR-1 (Rau et al., 2011). It was proposed that MBNL1 binding within the loop of pre-miR-1 disrupts LIN28 binding to this region and thereby promotes its processing by dicer (Rau et al., 2011). Our data argue against this model, as we show that (1) primary and mature miRNAs exhibit a parallel decrease in expression in heart tissues from DM1 mouse model and patient samples and (2) Mbn1 knockout mice do not show a significant change in miR-1 and many other miRNAs in heart. Instead, we provide evidence that a select set of miRNAs in DM1, including miR-1, is downregulated due to a reduced MEF2 transcriptional program.

The MEF2 paralogs are a conserved family of proteins that bind to a consensus DNA sequence CTA(A/T)4TAG in the promoter region of target genes (Molkentin and Olson, 1996). Although MEF2 proteins are expressed in various tissues, the expression of the MEF2 target genes in mouse is highest in skeletal muscle, heart, and brain (Potthoff and Olson, 2007). In addition to Gata4 and Tbx5, Mef2c is a key transcription factor required for direct reprogramming of cardiac fibroblasts into induced cardiomyocytes (Ieda et al., 2010; Qian et al., 2012). This study identifies loss of Mef2 activity as crucial to deregulation of many miRNAs and miRNAs in a DM1 cardiac cell culture model and heart tissue from DM1 mice. Reduced levels of Mef2a and Mef2c in response to transient expression of repeats in cultured cardiomyocytes argues for a direct effect of CUG expansion RNA in reduction of Mef2 levels. The results we obtained in DM1 heart tissue are in contrast to results from microarray studies showing increased expression of MEF2A and MEF2C in skeletal muscle from DM1 mice as well as other neuromuscular disorders (Bachinski et al., 2010) and suggest different pathogenic effects of CUG expansion RNA in heart and skeletal muscle. In summary, our data support a model in which nuclear accumulation of CUG expansion RNA in DM1 affects a MEF2-miRNA regulatory circuit such that reduced MEF2 activity results in loss of expression of its miRNA and mRNA targets in cardiac cells. The specific mechanism by which CUG expansion RNA affects mRNA and protein levels of MEF2 paralogs remains to be determined.

**EXPERIMENTAL PROCEDURES**

**Animal Models and Human Tissue Samples**

The tamoxifen (Tam)-inducible and heart-specific EpA960;MCM DM1 mouse model was described previously (Wang et al., 2007). CUG expansion RNA was induced...
in 2- to 4-month-old EpA960;MCM bitransgenic animals with a single injection or five consecutive daily intraperitoneal injections of 20 mg/kg Tam (Sigma-Aldrich). Tet-inducible CELF1 bitransgenic mice (TRECUGBP1/Myh6-rtTA) were previously described (Kalsotra et al., 2008). All experiments were conducted in accordance with the National Institutes of Health Guide for the Use and Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. Human tissue or RNA samples were provided by Drs. C. Thornton and T. Ashizawa, X. Wehrens, National Disease Research Interchange, and the University of Miami Tissue bank. DM1 samples were from a 50-year-old male (respiratory failure [RF]), 48-year-old female (1,500 repeats, RF), 55-year-old male (pneumonia [PN]), and fold change > 1.5) or between 72 hr and MCM; expression values were quantile normalized. Genes were selected that showed differential expression between 1 week and MCM (t test p < 0.01 and fold change > 1.5) or between 72 hr and MCM; expression values were clustered as previously described (Creighton et al., 2009).

**Protein and mRNA Expression Analysis**

Normal (non-DM1) and DM1 human heart tissue lysates were prepared and protein concentrations were determined by BCA assays (Pierce). A total of 50 μg of lysate from each sample was separated on 10% SDS-PAGE followed by western blot. MEF2A (54 kDa; Cell Signaling Technology) CELF1 (50 kDa; Cell Signaling Technology), and fold change were detected by using an HRP-chemiluminescence system (Pierce). Total RNA was prepared from heart samples using TRIzol. Steady-state mRNA expression was measured by qRT-PCR as previously described (Kalsotra et al., 2008). All individual pri-miRNA, miRNA, and mRNA qRT-PCR assays were performed using predesigned TaqMan primers and probes (Applied Biosystems) according to the manufacturer’s instructions.

**Alternative Splicing Assays**

Total RNA (0.3–0.5 μg) was used for RT-PCR as described elsewhere (Kalsotra et al., 2010). Primer sequences for detecting alternative splicing of Mef2a (a and b exons) and Mef2c (γ exon) are provided in Table S3. PSI values for the variable region were calculated with Kodak Gel logic 2200 and Molecular Imaging Software as ([inclusion band]/[inclusion band + exclusion band]) × 100.

**Cell Culture and Transfections**

HL-1 cells were cultured on gelatin (0.02%, w/v)/fibronectin (10 μg/ml) coated plates and maintained in Claycomb medium (JRH Biosciences) as previously
The HL-1 cells were infected in T25 flasks with tet-inducible control or Mef2c expressing virus in presence of 5 µg/ml polybrene. After 48 hr of recovery, the cells were switched to 1 µg/ml doxycycline-containing media for 12 hr. Next, the cells were transiently transfected with pBi-tet DT0-GFP or pBi-tet DT960-GFP plasmids with Lipofectamine 2000 using the manufacturer’s instructions. Cells were harvested 36 hr later in order to isolate total RNA or were fixed in 4% formaldehyde and permeabilized with 0.02% Triton X-100 in PBS. CUG transcripts were detected using (CAG)5-Cy3-labeled LNA probes (Exiqon) as described previously (Wang et al., 2007). Nuclei were stained with DAPI using Vectashield (Vector).

ChIP Assays
Mef2-ChIP was performed using the Imprint chromatin immunoprecipitation kit (Sigma-Aldrich) according to the manufacturer’s instructions with minor modifications. Three mouse hearts each from wild-type adults, MCM mice given tamoxifen for 1 week, and DM1 mice induced to express CUG repeat RNA for a week were collected in cold PBS, chopped into smaller pieces on ice and then incubated in 1% formaldehyde in PBS for 10 min at room temperature. Formaldehyde crosslinking was stopped by adding 10X glycine to a final concentration of 1X and incubating at room temperature for 5 min. Tissue was spun at 4°C at 220 g for 5 min and the remaining tissue pellet was rinsed twice in ice-cold PBS. Tissues were harvested and lysed to isolate nuclei in a hypotonic buffer, then re-suspended, lysed in lysis buffer, and sonicated in 15 ml tubes with Bioruptor UCD-200 Diagenode (ultrasound wave output power 250 W, 14 x 30 s) to yield chromatin size of 200–400 bp. ChIP was performed twice with 2µg of anti-RNA Pol II rabbit polyclonal (Santa Cruz Biotechnology, sc-9000) and anti-pan Mef2A and Mef2C goat polyclonal (Santa Cruz, sc-313), anti-Mef2c specific rabbit polyclonal (Cell Signaling Technology, sc-303) and 2µg of normal rabbit IgG (Santa Cruz, sc-2027) or goat IgG (Santa Cruz, sc-2028) as isotype controls. Coprocipitated DNA was then analyzed by qRT-PCR performed with SYBR green mix (Applied Biosystems). The primers used are listed in Table S3.

Statistics
Data are presented as mean ± SD. Statistical significance was determined with a two-tailed Student’s t test or one-way ANOVA followed by post hoc Tukey’s multiple range tests. A p value of less than 0.05 was considered significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.12.025.

AUTHOR CONTRIBUTIONS
A.K. designed research, performed the experiments, analyzed the data, and wrote the manuscript; R.K.S. performed experiments, analyzed data, and contributed to the manuscript; P.G. performed experiments and contributed to the manuscript; A.J.W. isolated heart tissues of EpA986/MCM animals and its controls and performed alternative splicing analysis; C.J.C. analyzed the microarray data and contributed to the manuscript; and T.A.C. supervised and designed research, analyzed the data, and wrote the manuscript.

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Figure S1. *miRNA*, EpA960 recombined allele, *Myh7* and *Acta1* expression in mouse and human DM1 and non-DM1 cardiomyopathies. (A) Steady state *miR*-23a and *miR*-23b levels during normal mouse heart development and in EpA960; MCM+Tam (DM1) mouse model were determined by real time quantitative PCR (qRT-PCR). Each bar represents fold change in expression (mean \( \pm \) SD) relative to wild type (Wt) embryonic day (E)14. ‘a’ represents significantly different from Wt adult; ‘b’ represents significantly different from Wt E14. Relative expression of cardiac hypertrophy markers (B) Relative expression of the EpA960 recombined allele at 72hr and 1wk following tamoxifen.
injection. (C) Myh7 and (D) Acta1 in DM1 mice, Calcineurin transgenic (CnA-Tg) mice and wild type mice after 8 weeks of Transverse Aortic Constriction (TAC). Individual bars represent fold change in Myh7 or Acta1 mRNA steady state levels (mean ± SD) from heart samples of EpA960;MCM+Tam mice relative to MCM+Tam controls, CnA-Tg mice relative to littermate controls (n=3) or from mice that underwent TAC surgery relative to shams. (E) Relative expression of miRNAs in failing human heart samples in comparison to non-disease controls based on qRT-PCR analysis. *P < 0.05.
Figure S2. Ingenuity Pathway Analysis showing genes affected in DM1 mouse hearts regulate mitochondrial function.

Ingenuity analyses indicated a canonical pathway involving mitochondrial dysfunction as over-represented categories (P value = 5.36E-08) in DM1 mouse hearts as compared to controls. Pink/red color of molecules indicates down regulation of genes analyzed in our study, with darker shades of pink represents levels of gene down regulation. Green color indicates up regulated genes. Grey color indicates no change. Forty-five of 174 genes in the pathway were affected in heart tissue from the DM1 mouse model.
Figure S3. Mef2A and Mef2c splicing and steady state level expression analysis. (A) Alternative exons in Mef2a (α & β exons) and Mef2c (γ exon) show no significant change in PSI values after 1 wk of CUG<sup>exp</sup> RNA expression compared to MCM controls. qRT-PCR analysis of Mef2a and Mef2c mRNA steady state levels in, (B) TgCELF1 mice relative to MHCrtTA mice given doxycycline (dox), (C) Mbnl<sup>+/+</sup> mice relative to Mbnl<sup>Δ3/Δ3</sup> mice and (D) Unaffected human control and failing hearts. Each bar represents fold change in
expression (mean ± SD). Data is normalized relative to ribosomal protein L30 (Rpl30).
n=3. (E) Alternative splicing of MEF2A and MEF2C in unaffected or DM1 hearts.
Figure S4. Mef2 proteins bind to genomic regions surrounding several miRNAs in heart. Chromatin Immunoprecipitation (ChIP) was performed using a RNA Pol II, Mef2 or control IgG on wild type adult mouse hearts as described in the Methods section. PCR of the DNA input, RNA Pol II or Mef2 immunoprecipitate, and IgG control precipitate was performed using primers flanking Mef2 consensus sites on each of the miRNAs. The primers used for PCR assays span the Mef2 binding sites in target (A) mRNAs or (B) primary miRNAs.
SUPPLEMENTAL REFERENCES


