Cytoplasmic CUG RNA Foci Are Insufficient to Elicit Key DM1 Features

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Abstract

The genetic basis of myotonic dystrophy type I (DM1) is the expansion of a CTG tract located in the 3’ untranslated region of DMPK. Expression of mutant RNAs encoding expanded CUG repeats plays a central role in the development of cardiac disease in DM1. Expanded CUG tracts form both nuclear and cytoplasmic aggregates, yet the relative significance of such aggregates in eliciting DM1 pathology is unclear. To test the pathophysiology of CUG repeat encoding RNAs, we developed and analyzed mice with cardiac-specific expression of a beta-galactosidase cassette in which a (CTG)90 repeat tract was positioned 3’ of the termination codon and 5’ of the bovine growth hormone polyadenylation signal. In these animals CUG aggregates form exclusively in the cytoplasm of cardiac cells. A key pathological consequence of expanded CUG repeat RNA expression in DM1 is aberrant RNA splicing. Abnormal splicing results from the functional inactivation of MBNL1, which is hypothesized to occur due to MBNL1 sequestration in CUG foci or from elevated levels of CUG-BP1. We therefore tested the ability of cytoplasmic CUG foci to elicit these changes. Aggregation of CUG RNAs within the cytoplasm results both in MBNL1 sequestration and in approximately a two fold increase in both nuclear and cytoplasmic Cug-bp1 levels. Significantly, despite these changes RNA splice defects were not observed and functional analysis revealed only subtle cardiac dysfunction, characterized by conduction defects that primarily manifest under anesthesia. Using a human myoblast culture system we show that this transgene, when expressed at similar levels to a second transgene, which encodes expanded CTG tracts and facilitates both nuclear focus formation and aberrant splicing, does not elicit aberrant splicing. Thus the lack of toxicity of cytoplasmic CUG foci does not appear to be a consequence of low expression levels. Our results therefore demonstrate that the cellular location of CUG RNA aggregates is an important variable that influences toxicity and support the hypothesis that small molecules that increase the rate of transport of the mutant DMPK RNA from the nucleus into the cytoplasm may significantly improve DM1 pathology.


Editor: Katrina Gwinn, Baylor College of Medicine, United States of America

Received May 12, 2008; Accepted October 16, 2008; Published December 18, 2008

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Funding: We gratefully acknowledge support from the Dr. Hauschneckt for funds that partially supported this work. This work was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 (5R01NS050861, 1R01NS060839) from the National Center for Research Resources, National Institutes of Health (NHRI).

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Myotonic dystrophy 1 (DM1) is a multi-system disorder characterized by skeletal myopathy and cardiac disease [1]. Sudden cardiac failure is one of the main causes of death in DM1 patients. Cardiac symptoms include variable conduction disorders and wall motion abnormalities [2–6]. First degree atrioventricular (AV) block and intraventricular conduction disorders are seen in ~75% of DM1 patients [2]. Progressive deterioration of the conduction system resulting in complete AV block or ventricular arrhythmias are primarily responsible for sudden cardiac death [3,4]. Although conduction disorders predominate in DM1, decreased ventricular systolic and diastolic functions, and hypertrophic and dilated cardiomyopathy, have been reported in severely affected patients [5–8]. Histological abnormalities include myofibrillar loss, fibrosis and fatty infiltration of both the working myocardium and the specialized conduction system. Electron microscopic examination shows aberrant Z lines and mitochondrial abnormalities in DM1 hearts [9].

The genetic defect in DM1 is the expansion of a CTG repeat tract on chromosome 19q13.3. The repeat expansion is located in the 3’ untranslated region of a protein kinase gene, DMPK, and is found 5’ of a homeodomain-encoding gene, SIX5 [10–13]. CTG tract size is a strong predictor of cardiac involvement, particularly for electrophysiologic conduction, and wall motion abnormalities. Small expansions of 50–100 repeats produce a mild form of DM1 characterized primarily by the development of cataracts late in adult life. A multi-system adult onset form of the disease manifesting with cardiac disease occurs in a range of 250–500...
repeats. Progressive increase in CTG tract size, to lengths greater than 1500 repeats, results in increased incidence and severity of the cardiac phenotype [4,5]. Three non-exclusive molecular defects hypothesized to contribute to DM1 pathology are (i) decreased DMPK levels resulting from aberrant nuclear accumulation of the mutant DMPK RNA [14,15] (ii) decreased SIX3 levels occurring as a consequence of chromatin condensation that occurs in the vicinity of the expanded CTG tract [16–18] and (iii) intrinsic toxicity of the expanded CUG tracts [19].

We have previously shown that both Dmpk−/− and Dmpk−/+ mice demonstrate PR prolongation or first degree heart block, while Dmpk+/− mice exhibit a more severe phenotype consisting of both second and third degree heart block [20–22]. However, histological defects and wall motion abnormalities are not detected in these animals and the life-span of wild-type and mutant Dmpk mice is not significantly different. Structure-function analysis of Six5+/− cardiac muscle demonstrates that reduced Six5 levels result in mild infra-Hisian conduction delay, increased left ventricular end diastolic dimension, and ventricular hypertrophy [23]. As reduction in Dmpk and Six5 levels does not completely recapitulate the severity of DM1 cardiac pathology, these data suggest that toxic effects associated with the expression of CUG repeats play a prominent role in the etiology of DM1 heart disease. Consistent with a key role for expanded CUG repeat RNA in DM1 cardiac pathophysiology, indoluble expression of high levels ∼960 interrupted CTG repeats located in the DMPK 3′UTR results in arrhythmias and cardiomyopathy that often lead to death of the transgenic animals within a few weeks after induction of the transgene [24]. In these experiments both elevated Cug-bp1 levels and aggregation of Mbnl1 in intra-nuclear RNA foci are documented in conjunction with aberrant splice site selection in a set of physiologically important RNAs [24]. Thus, as expression of expanded CUG repeats elicits key features of DM1 cardiac pathology, therapeutic strategies will require identification of mechanisms that will allow such RNAs to be rendered inert.

Here, we demonstrate that the cellular location of CUG RNA aggregates is a variable that influences the development and severity of DM1 cardiac pathology. In this study, we developed transgenic mice with cardiac-specific expression of a β-galactosidase cassette in which a (CTG)400 repeat tract is located 3′ of the termination codon of the β-galactosidase gene and 5′ of the bovine growth hormone poly A sequence. In these animals RNAs encoding expanded CUG repeats were found to aggregate exclusively within the cytoplasm of cardiomyocytes. Both in DM1 cells and in transgenic mice demonstrating cardiac specific expression of expanded CTG tracts located in the DMPK 3′UTR, aberrant RNA splicing is observed in conjunction with the aggregation of Mbnl1 in the nuclear CUG foci and increased steady-state levels of Cug-bp1 [24–26]. We therefore tested the ability of cytoplasmic CUG foci to elicit these changes. We observe both sequestration of Mbnl1 in the cytoplasmic CUG aggregates and approximately a two-fold elevation in the levels of Cug-bp1 in cardiomyocytes. Importantly, these defects did not result in abnormal RNA splicing and caused only a mild cardiac pathology, which manifests primarily as conduction defects under anesthesia. These results demonstrate that (i) CTG tracts expressed in a context independent manner can elicit elevated Cug-bp1 levels, (ii) a two fold elevation of Cug-bp1 levels is insufficient to dysregulate splice site choice in the adult mouse heart and (iii) aggregation of Mbnl1 in CUG foci per se may not be sufficient to inactivate Mbnl.

The relative lack of toxicity of cytoplasmic CUG RNA aggregates is unlikely to be a consequence of low expression levels, as an independent set of experiments carried out in human myoblasts demonstrate that when this transgene is expressed at similar levels to a second transgene in which the expanded CTG tract was expressed in the context of the DMPK 3′UTR, it is unable to dysregulate RNA splicing. Consistent with our study, previous experiments in mouse myoblast cultures demonstrate that CTG tracts expressed in the context of the DMPK 3′UTR are no longer able to dysregulate myoblast differentiation when this cassette was re-engineered to encode the woodchuck post transcriptional element that served to localize the CUG encoding RNA within the cytoplasm [27]. These data therefore demonstrate that when expanded CUG tracts, expressed in a context independent manner or in the context of the DMPK 3′UTR, localize in the cytoplasm, they are unable to cause aberrant RNA splicing or significant pathology, when assessed in myoblast cultures or in the context of the adult mouse. Thus our data support the therapeutic use of small molecules that increase the transport of the mutant DMPK RNA from the nucleus into the cytoplasm as a means of greatly ameliorating DM1 pathology in vivo.

Results

DM1 myoblasts and fibroblasts contain both nuclear and cytoplasmic CUG foci

Both primary cultures and SV40 transformed DM1 fibroblasts and myoblasts show CUG RNA foci in the nucleus and cytoplasm [Figure 1 and Supplementary Figure S1 (Panels A–E)]. SV40 transformation does not significantly alter the localization of CUG foci, as the percent of DM1 fibroblasts, which contain both nuclear and cytoplasmic foci, did not vary appreciably in untreated cultures and in SV40 immortalized lines [Figure 1; Table 1].

Construction and analyses of α-MHC-LacZ-(CTG)400 mice

To characterize the pathological effects intrinsic to the expression of expanded CTG repeat tracts in cardiac muscle, we built a transgenic cassette encoding the gene for β-galactosidase (LacZ) followed by a tract of ∼400 uninterrupted CTG repeats, which was cloned in a linker sequence and inserted between the LacZ termination codon and the bovine growth hormone polyadenylation (BGH-PolyA) sequence. A 5.5 kb α-myosin heavy chain (α-MHC) promoter, which encodes the first three untranslated exons of α-MHC, was used to drive specific expression of the LacZ-(CTG)400 cassette in the myocardium of embryonic atria and in adult mouse atria and ventricles [28; Figure 2; Panel A].

Consistent with the instability, which characterizes uninterrupted CTG tracts [29,30], the (CTG)400 repeats tract showed a marked propensity to either delete or decrease in length when the plasmid encoding the transgenic cassette was propagated in bacteria at 37°C [Figure 2; Panel B]. The CTG tract was mutation-free when sequenced from each end to a distance of ∼300 bp, after which significant compressions were observed.

α-MHC-LacZ-(CTG)400 cassettes were injected into fertilized C57BL/6J mouse eggs to generate two independent lines. A α-MHC-LacZ cassette with no CTG tracts [α-MHC-LacZ-(CTG)0] was injected in parallel to develop control lines. Southern blot analyses of tail tip DNA from the progeny of both α-MHC-LacZ-(CTG)400 founders derived after approximately one year of breeding are shown in Figure 2; Panel C. Comparison of the two α-MHC-LacZ-(CTG)400 lines demonstrate that the lines showed deletions which ranged in length from ∼50–70 and ∼150–170 CTG repeats. However the majority of the CTG tracts contained ∼300–380 repeats with a significant fraction showing expansions that occurred primarily to lengths of ∼700–730 and ∼800 CTG repeats.

Consistent with the average CTG tract sizes observed in the transgenic mice, analysis of RNA isolated from hearts of α-MHC-
LacZ mice and α-MHC-LacZ-(CTG)400 mice by Northern blots, showed transcript sizes of 3 and 4.2 kb when probed with β-galactosidase sequences [Figure 2; Panel D]. Progeny from the two founder α-MHC-LacZ-(CTG)400 lines, demonstrate different levels of LacZ-(CUG)400 RNA and are therefore denoted as α-MHC-LacZ-(CTG)400TG high and α-MHC-LacZ-(CTG)400TG low in the text.

Mbnl1 sequesters in cytoplasmic CUG RNA foci in α-MHC-LacZ-(CTG)400 cardiomyocytes

To examine the behavior of LacZ-(CUG)400 RNAs, we isolated cardiomyocytes from transgenic hearts and used fluorescence in situ hybridization (FISH) to visualize the location of the expanded CUG tracts with a (CAG)10-Cy3 probe (red signal; Panels b, k, n, q & w). Transcripts containing expanded CUG repeats are not observed in the normal cardiomyocytes and normal myocytes (b & e), respectively. Merged images of DAPI and (CAG)10-Cy3 stains show CUG RNA foci as red signals (i, l, o, r, u & x) within the nucleus and in the cytoplasm in DM1 cardiomyocytes and myocytes. The percent of DM1 cardiomyocytes and myocytes containing both nuclear and cytoplasmic foci are tabulated in Table 1. Images of CUG RNA foci in additional DM1 cardiomyocytes and myocytes are shown in supplementary Figure S1 (Panels A–E).

Table 1. DM1 fibroblasts and myoblasts contain both nuclear and cytoplasmic CUG foci.

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<tr>
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<td>68</td>
<td>45</td>
<td>23 (33.82%)</td>
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<tr>
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<td>93</td>
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<td>53</td>
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doi:10.1371/journal.pone.0003968.g001

doi:10.1371/journal.pone.0003968.t001

Figure 1. DM1 fibroblasts and myoblasts contain both nuclear and cytoplasmic CUG foci. Panel A: Nuclear DAPI staining of normal and DM1 fibroblasts and myoblasts, either untreated or immortalized with SV40, are shown in Panels a, d, g, m, p, s & v. DMPL transcripts encoding the expanded CUG tracts were detected by hybridization with a (CAG)10-Cy3 probe (red signal; Panels h, k, n, q, t & w). Transcripts containing expanded CUG repeats are not observed in the normal fibroblasts and normal myoblasts (b & e), respectively. Merged images of DAPI and (CAG)10-Cy3 stains show CUG RNA foci as red signals (i, l, o, r, u & x) within the nucleus and in the cytoplasm in DM1 fibroblasts and myoblasts. The percent of DM1 fibroblasts and myoblasts containing both nuclear and cytoplasmic foci are tabulated in Table 1. Images of CUG RNA foci in additional DM1 fibroblasts and myoblasts are shown in supplementary Figure S1 (Panels A–E).
To test if CUG-RNA aggregates aberrantly sequester the alternative splice factor, Mbnl1, we stained both α-MHC-LacZ and α-MHC-LacZ-(CTG)400 cardiomyocytes and normal human and DM1 myoblasts with anti-MBNL1 (MB1a) monoclonal antibodies [31]. Co-localization studies demonstrate that Mbnl1 aberrantly sequesters within the cytoplasmic CUG RNA foci in α-MHC-LacZ-(CTG)400 cardiomyocytes [Figure 4; Panel A]. Quantitation of the sequestered Mbnl1 demonstrates that the percent of Mbnl1 sequestered in the cytoplasmic foci in α-MHC-LacZ-(CTG)400 cardiomyocytes is not significantly different (6.65%) from the percent of MBNL1 sequestered within the nuclear and cytoplasmic foci in DM1 myoblasts (8.01%) [p = 0.37, Figure 4; Panel B & Table 2]. The specificity of MBNL1 (MB1a) monoclonal antibody was verified by immunofluorescence using cardiomyocytes derived from Mbnl1−/− mice [32, Supplementary Figure S2]. Thus these results demonstrate that cytoplasmic CUG foci, although more diffuse in appearance, can effectively sequester Mbnl1 in vivo.

Cug-bp1 levels are elevated in α-MHC-LacZ-(CTG)400 cardiomyocytes
To test if expression of LacZ-(CUG)400 RNAs can elicit a change in steady-state Cug-bp1 levels, we measured the steady-state levels of Cug-bp1 in tissue lysates derived from hearts of 6 months old α-MHC-LacZ-(CTG)400TG high, α-MHC-LacZ-(CTG)400TG low and α-MHC-LacZ mice. Three independent western blot analyses in which hearts from two mice of each genotype were analyzed demonstrate a 2.5 fold increase in Cug-bp1 levels in α-MHC-LacZ-(CTG)400TG high and a 1.6 fold increase in α-MHC-LacZ-(CTG)400TG low mice. The fold changes in steady-state Cug-bp1 levels were not significantly different when either 6 μg or 10 μg of proteins were sampled [Figure 5; Panel A]. In these experiments no significant alteration in steady-state Mbnl1 levels were detected in α-MHC-LacZ and α-MHC-LacZ-(CTG)400TG high mice [Figure 5; Panel B]. To test if nuclear Cug-bp1 levels were altered in α-MHC-LacZ-(CTG)400 mice, we measured Cug-bp1 protein levels in the cytoplasmic and nuclear

Figure 2. Characterization of α-MHC-LacZ-(CTG)400 mice. Panel A: The α-MHC-LacZ-(CTG)400 transgene encoding the α-myosin heavy chain promoter (α-MHC) used to drive cardiac specific expression of the β-galactosidase (LacZ) gene followed by a CTG tract of ~400 repeats and the bovine growth hormone polyA (BGH-PolyA) sequence is shown. Panel B: Restriction digestion of plasmids encoding the α-MHC-LacZ-(CTG)400 sequences with SfiI, which allows excision of the CTG repeat tract, demonstrates the instability of the CTG repeats when propagated in E. coli at 37°C. Panel C: Southern blot analysis of mouse tail-clip DNA digested with PvuII. The 280 bp probe used for hybridization (Panel A) is shown. The majority of the detected bands contained 350–380 CTG repeats in α-MHC-LacZ-(CTG)400TG high (band intensities ~76%) or 300–350 CTG repeats in α-MHC-LacZ-(CTG)400TG low (band intensities ~80%) in tail clip DNAs. Panel D: Northern blot analysis of RNA derived from α-MHC-LacZ-(CTG)400 and α-MHC-LacZ mouse hearts probed with β-galactosidase and Gapdh sequences is shown.

doi:10.1371/journal.pone.0003968.g002
fractions of heart tissue derived from α-MHC-LacZ, α-MHC-LacZ-(CTG)400 high, and α-MHC-LacZ-(CTG)400 low animals. In these experiments Cug-bp1 was found to localize primarily in the cytoplasm in both α-MHC-LacZ and α-MHC-LacZ-(CTG)400 mice. Cug-bp1 levels were elevated, 2.7 and 2.4 fold, and 1.6 and 1.2 fold in the cytoplasm and in the nucleus of MHC-LacZ-(CTG)400 and α-MHC-LacZ-(CTG)400 high/low heart tissue sections [red signals; e, f (40 × magnification) and a, i (120 × magnification)]. Transcripts containing expanded repeats are not observed in cardiomyocytes and heart sections of α-MHC-LacZ mice and in normal human myoblasts [Panel A; a, d and Panel B; d, g]. >400 cells were examined in both α-MHC-LacZ-(CTG)400 cardiomyocyte preparations and in α-MHC-LacZ-(CTG)400 cardiac sections.

doi:10.1371/journal.pone.0003968.g003

Figure 3. CUG foci form exclusively in the cytoplasm of α-MHC-LacZ-(CTG)400 cardiomyocytes. Panel A: Nuclear DAPI staining of cardiomyocytes derived from α-MHC-LacZ, α-MHC-LacZ-(CTG)400 mice and normal human and DM1 myoblasts is shown. Transcripts encoding the expanded CUG tracts were detected by hybridization with a (CAG)10-Cy3 probe (red signal). CUG foci are observed in the cytoplasm in α-MHC-LacZ-(CTG)400 cardiomyocytes (b, d) and in both the cytoplasm and nucleus of DM1 myoblasts (e) (120 × magnification). Panel B: Nuclear DAPI staining of heart sections of α-MHC-LacZ, α-MHC-LacZ-(CTG)400TG high, and α-MHC-LacZ-(CTG)400TG low are shown. CUG foci are observed in the cytoplasm in both α-MHC-LacZ-(CTG)400TG high, and α-MHC-LacZ-(CTG)400TG low heart tissue sections [red signals; e, f (40 × magnification) and a, i (120 × magnification)].

RNA splicing is not dysregulated in α-MHC-LacZ-(CTG)400 hearts

As the cytoplasmic LacZ-CUG foci aggregate Mbnl1 and elicit elevated steady-state levels of Cug-bp1, we studied the effect of LacZ-(CUG)400 RNA expression on RNA splice site choice. We examined the pattern of alternative splicing of cardiac troponin T (Tnnt2), Z-band alternatively spliced PDZ-motif protein (Zasp), alpha-actinin-2 associated LIM protein (Alp) and M-line Titin (m-Titn) [33-35] RNAs. These RNAs are aberrantly spliced in adult DM1 hearts and retain the pattern of splicing observed in newborns [36]. No significant change in the splicing pattern of these RNAs is observed in heart tissue of either the α-MHC-LacZ-(CTG)400TG high or α-MHC-LacZ-(CTG)400TG low mice when compared to control α-MHC-LacZ mice. In all cases, the relative percentages of the detected isoforms for each gene did not vary significantly for each of the two amplification conditions tested [Figure 6 & Table 3].

Expression of LacZ-(CUG)400 RNAs at levels equivalent to that of a DMPK mini gene encoding 300 CTG repeats does not result in aberrant RNA splicing in human myoblasts

To examine if low expression levels are responsible for the lack of toxicity of LacZ-(CUG)400 RNAs in α-MHC-LacZ-(CTG)400 mice, we studied a set of expression vectors in human myoblast cultures in which the cytomegalovirus (CMV) promoter was used to express cassettes encoding a DMPK minigene encoding either 5 or 300 interrupted CTG repeats [DMPK 11-15(CTG)5 or 300], the
green fluorescent protein linked to the DMPK 3'UTR containing either 5 or 400 CTG repeats [GFP-DMPK 3'UTR(CTG)5 or 400], or the β-galactosidase gene containing no repeats or 400 repeats [LacZ-(CTG)0 or 400] [Figure 7; Panel A]. Expression of constructs encoding both the DMPK 11-15 minigene and GFP-DMPK 3'UTR with the expanded CTG repeats resulted in nuclear foci and aberrant RNA splicing. All constructs that did not contain the expanded CTG tracts did not dysregulate RNA splicing. Expression of the LacZ-(CTG)400 construct formed cytoplasmic CUG foci but did not alter splice site selection in IR and cTNT RNAs [Figure 7; Panels B–C and Table 4]. RT-PCR analyses of the steady-state expression levels of the β-galactosidase cassette encoding 400 CTG tracts and the DMPK minigene encoding 300 CTG repeats, which are approximately 4.3 and 2.2 kb in length.
respectively, demonstrate that the expression levels achieved by these casettes was not significantly different [p = 0.479, Figure 8; Panels A-B and Table 5]. To further confirm that comparable expression levels of LacZ-(CUG)300, and DMPK 11-15(CUG) 300 RNAs were assayed in these experiments, we quantitated the amounts of LacZ-

**Table 2. Distribution of MBNL1 in α-MHC-LacZ-(CTG)400 cardiomyocytes and DM1 myoblasts.**

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<thead>
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<th>Cell type</th>
<th>Cell #</th>
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<th>p-value (Foci)</th>
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(c)represents pair wise comparison; p-value (Student’s t-test) denotes no significant difference in MBNL1 sequestered in foci of α-MHC-LacZ-(CTG)400TGhigh and DM1 myoblasts. doi:10.1371/journal.pone.0003968.t002

Mitochondrial defects are observed α-MHC-LacZ-(CTG)400 heart tissue

No gross abnormalities in the cardiac muscle structure were observed in H & E sections of α-MHC-LacZ-(CTG)400 mice (data not shown). Electron microscopy showed disarrayed cristae in mitochondria in some sections of both α-MHC-LacZ-(CTG)400TGhigh and α-MHC-LacZ-(CTG)400TGlow mice but not in α-MHC-LacZ mice [Figure 9]. Thus consistent with the lack of RNA splice defects in α-MHC-LacZ-(CTG)400 mice, these results demonstrate that LacZ-(CUG)400 RNAs are unable to elicit significant cardiac pathology in vivo.

Discussion

Myotonic dystrophy is a multi-system disorder, characterized by aberrant RNA splicing, which results from the expansion of a CTG tract located in the 3'UTR of **DMPK**. An important mediator of DM1 pathology is the mutant **DMPK** RNA encoding the expanded CUG tracts [24,37–39]. Thus a central aspect of designing therapeutic interventions for this disease is to determine how to alter such toxic RNAs into benign or relatively inert macromolecules. RNAs encoding expanded CUG repeats form both nuclear and cytoplasmic aggregates or foci in DM1 cells. The relative toxicity of such aggregates both in terms of evoking aberrant RNA splicing and in eliciting DM1 pathophysiology in vivo is currently unknown. In this study we describe the behavior of expanded CTG tracts expressed in the context of the β-galactosidase gene under the control of the α-myosin heavy chain promoter in mouse hearts. LacZ-(CUG)400 RNAs form aggregates exclusively in the cytoplasm of cardiomyocytes in transgenic mice. Significantly, the cytoplasmic LacZ-CUG RNA aggregates are unable to dysregulate splice site choice in mouse hearts and result only in mild cardiac dysfunction. Our results therefore support a therapeutic strategy aimed at the identification of small molecules that facilitate effective and rapid transport of toxic CUG RNAs from the nucleus into the cytoplasm as a means of markedly reducing the toxicity of such RNAs.

Several lines of evidence demonstrate that mutant RNAs encoding expanded CUG repeat tracts embedded in the DMPK 3'UTR, which aggregate within the nucleus, facilitate the development of DM1 pathology. Szczesniak and colleagues have shown that expression of expanded CTG tracts in the context of the human DMPK gene results both in nuclear foci and the development of DM1 pathology in mice [38]. In this study, the severity of the phenotype was influenced both by tract size and expression levels. Specifically, 300 CUG repeats were found to be the minimum repeat tract length at which an overt pathology was detected in mice. Transgene expression levels were a second variable in this study, as homozygous animals were more severely...
affected than hemizygous mice. Nonetheless, not all of the animals that expressed CUG foci in the nucleus demonstrate a DM1 phenotype. Such differences were attributed by the authors to possible variations in the pattern of transgene expression during development or differences in RNA stability [38]. Consistent with these earlier results, inducible expression of high levels of interrupted CUG repeat tracts expressed in the context of the DMPK 3'UTR, under the control of a strong ubiquitous promoter, has also been shown to result in the rapid aggregation of CUG repeats within the nucleus and the development of severe

Figure 5. α-MHC-LacZ-(CTG)400 mice show increased steady-state levels of Cug-bp1. Panels A-B: Protein extracts were prepared from α-MHC-LacZ, α-MHC-LacZ-(CTG)400TG<sup>high</sup>, and α-MHC-LacZ-(CTG)400TG<sup>low</sup> mouse hearts and 6 or 10 μg of the total proteins from the tissue extracts were resolved on SDS-PAGE followed by Western blot analyses and immunostaining with CUG-BP1 and MBNL1 monoclonal antibodies (mAbs), respectively. The blots were re-probed for GAPDH using anti-GAPDH polyclonal antibodies as an internal control. The experiments were carried out in triplicate and mean values of steady-state Cug-bp1 and Mbnl1 levels are shown. Panel C: Cytoplasmic and nuclear proteins extracts (10 μg) from α-MHC-LacZ, α-MHC-LacZ-(CTG)400TG<sup>high</sup>, and α-MHC-LacZ-(CTG)400TG<sup>low</sup> mouse hearts were resolved on SDS-PAGE followed by Western blot analyses and immunostaining with CUG-BP1 mAb. The blots were re-probed for TATA binding protein (TBP), and for GAPDH, which were used as nuclear and cytoplasmic markers respectively. The experiments were carried out in triplicate and mean values of steady-state Cug-bp1 levels are shown. doi:10.1371/journal.pone.0003968.g005

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<td>67.9±4.7</td>
<td>65.5±3.1</td>
<td>67.6±4.1</td>
<td>69.1±5.3</td>
<td>91.6±0.7*</td>
<td>89.7±1.3*</td>
</tr>
<tr>
<td>m-Ttn</td>
<td>XM_130312.Xml 5</td>
<td>4</td>
<td>3</td>
<td>48.7±1.2</td>
<td>47.6±1.3</td>
<td>48.2±1.3</td>
<td>47.4±0.9</td>
<td>98.6±0.4*</td>
<td>98.4±0.5*</td>
</tr>
</tbody>
</table>

Asterisk (*) represents significant differences from wild type (WT) adult (Student’s test; p<0.05). Tnt2, cardiac troponin T; Alp, alpha-actinin-2 associated LIM protein; Zasp, Z-band alternatively spliced PDZ-motif protein; m-Ttn, M-line Titin; LacZ, α-MHC-LacZ-(CTG)<sub>400</sub>; TG<sup>high</sup>, α-MHC-LacZ-(CTG)<sub>400</sub>TG<sup>high</sup>; TG<sup>low</sup>, α-MHC-LacZ-(CTG)<sub>400</sub>TG<sup>low</sup>. doi:10.1371/journal.pone.0003968.t003

Figure 6. Aberrant splicing is not observed in α-MHC-LacZ-(CTG)400 hearts. Total RNA isolated from adult α-MHC-LacZ-(CTG)400 and adult α-MHC-LacZ hearts and wild-type postnatal day 1 and day 2 mouse hearts was subjected to RT-PCR analysis using the Tnnt2, Alp, Zasp and m-Ttn primers as described in Methods. Gapdh RNA was amplified in parallel as an internal control. The experiments were carried out in triplicate and the results are tabulated in Table 3. doi:10.1371/journal.pone.0003968.g006
Figure 7. Expression of LacZ-(CUG)$_{400}$ RNAs is insufficient to dysregulate IR and cTNT splicing in human myoblasts. Panel A: DMPK 11-15(CTG)$_5$ or 300 (a), GFP-DMPK 3’UTR (CTG)$_5$ or 400 (b) and LacZ-(CTG)$_{0}$ or 400 (c) cassettes under the transcriptional control of the cytomegalovirus (CMV) promoter are shown. Panel B: Nuclear DAPI staining of human normal myoblasts expressing DMPK11-15(CTG)$_300$ (a), DMPK 11-15(CTG)$_{300}$ (b), GFP-DMPK 3’UTR(CTG)$_5$ (c), GFP-DMPK 3’UTR(CTG)$_{400}$ (d), LacZ-(CTG)$_{0}$ (e), and LacZ-(CTG)$_{400}$ (f) cassettes are shown. The mutant transcripts encoding the expanded CUG tracts were detected by hybridization with a (CAG)$_{10}$-Cy3 probe. CUG RNA foci are observed primarily within the nucleus in normal myoblasts expressing DMPK11-15(CTG)$_{300}$ (red signal; b) and GFP-DMPK 3’UTR (CTG)$_{400}$ (red signal; d). CUG RNA foci are observed in the cytoplasm (red signal; f) in normal myoblasts expressing the LacZ-(CTG)$_{400}$ cassette. Normal myoblasts expressing DMPK11-15(CTG)$_{300}$ (a), GFP-DMPK 3’UTR(CTG)$_5$ (c), and LacZ-(CTG)$_{0}$ (e) do not show CUG RNA foci.
Specifically, structural analysis did not demonstrate gross abnormalities. Panel C: IR and cTNT RNA splicing in myoblasts expressing the indicated cassettes are shown. Synthesized cDNAs (150 ng) were subjected to RT-PCR analysis using the IR and cTNT primers described in Methods. GAPDH RNA was amplified in parallel as an internal control. The experiments were carried out in triplicate. Representative panels are shown in Panel C and the results are tabulated in Table 4. 

d, and LacZ-(CTG)9 (e) constructs did not show RNA foci. Panel C: IR and cTNT RNA splicing in myoblasts expressing the indicated cassettes are shown. Synthesized cDNAs (150 ng) were subjected to RT-PCR analysis using the IR and cTNT primers described in Methods. GAPDH RNA was amplified in parallel as an internal control. The experiments were carried out in triplicate. Representative panels are shown in Panel C and the results are tabulated in Table 4. 

doi:10.1371/journal.pone.0003968.g007

DM1 pathology in mice [24,39]. CUG expansions that are expressed in a context other than the DMPK 3’UTR show more variable results. Expression of high levels of RNAs encoding ~250 CUG tracts located in the 3’UTR of the human skeletal actin gene cause both intra-nuclear foci and DM1 pathophysiology in mouse skeletal muscles [37]. In contrast, expression of ~162 CUG repeats embedded in sequences that contain ~100 bps of DMPK 3’UTR sequences in flies showed nuclear foci but no pathology [40]. In a second study in flies, 480 interrupted CUG tracts expressed as a non-coding transcript allowed both the development of nuclear foci and pathology in several tissues [41]. In a third study, inducible expression of interrupted CUG tracts, varying in length from 16 to 480 repeats, was studied in various transgenic insertion contexts in flies. In this set of experiments several fly strains showed nuclear foci but only one strain of transgenic flies showed significant pathology [42]. It is currently unclear why such variability is observed in flies; however CTG tract sizes, expression levels and context of expression may all contribute to the differences in the observed phenotypes. These data demonstrate that although nuclear foci are not sufficient to produce a DM1 phenotype, in all cases in which DM1 pathology develops nuclear foci are observed. Thus taken together these data support the hypothesis that nuclear foci are required for DM1 pathology to manifest, but may not under some circumstances be sufficient to produce an overt phenotype, when either the CTG tract length, RNA expression levels, stability or context are less than optimal.

A noteworthy exception to this rule is the production of a DM1 phenocopy that results from the inducible expression of GFP sequences linked to the normal DMPK 3’UTR in mice [43]. No foci are observed in this study as the transgene encodes only 5 CTG repeats. Surprisingly, both severe cardiac and skeletal muscle pathology in conjunction with RNA splice defects are observed in these animals [43]. However, both our current study and those of others, demonstrate that expression of similar transgenes, in which GFP sequences are linked to the normal DMPK 3’UTR, in myoblast cell cultures is insufficient to cause aberrant RNA splicing or dysregulate myoblast differentiation [Figure 7, 44]. Thus the precise mechanism that underlies both the heart and skeletal muscle phenotypes observed in this phenocopy has yet to be completely understood. As DM1 patients are characterized by expanded CTG tracts, it is likely that the mechanistic basis for the pathology observed in this mouse strain differs in important ways from that observed in DM1 patients.

As noted above, a study by Wang and colleagues demonstrates that inducible expression of 960 interrupted CUG repeats located in the DMPK 3’UTR sequence results in the development of nuclear foci concurrent with arrhythmias, cardiomyopathy, cystolic and diastolic dysfunction and aberrant splicing [24]. In this study, we examined cardiac specific expression of 400 uninterrupted CUG repeats located in the 3’ of the β-galactosidase gene. In contrast to the results obtained by Wang et al., LacZ-(CTG)400 RNAs aggregate exclusively in the cytoplasm and do not result in aberrant splice site selection in several RNAs implicated in DM1 including Tmnt2, Alp, Zasp and m-Titin [Figure 6]. Consistent with the lack of splicing defects mild cardiac dysfunction was observed in α-MHC-LacZ-(CTG)400 mice. Specifically, structural analysis did not demonstrate gross abnor-
Figure 8. Quantitation of the steady-state levels of LacZ-(CUG)_{400} and DMPK 11-15(CUG)_{300} RNAs. Panels A–B: RT-PCR analyses of the steady-state expression levels of LacZ-(CTG)_{0}, LacZ-(CTG)_{400}, and DMPK 11-15(CTG)_{5}, DMPK 11-15(CTG)_{300} cassettes are shown. Synthesized cDNA (100 ng) from normal myoblasts expressing LacZ-(CTG)_{0} or 400 and DMPK 11-15(CTG)_{5} or 300 were subjected to RT-PCR analysis. GAPDH RNA was amplified in parallel as an internal control. The experiments were carried out in triplicate and the results are tabulated in Table 5. Relative steady-state expression levels of the LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} cassettes were not significantly different (p = 0.479).

Panels C–F: Real-time PCR analysis of serial dilutions of plasmid DNAs encoding LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} sequences and of LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} cDNAs is shown. PCR reactions were carried using 10^{-2} to 10^{-6} fmoles of plasmid DNAs encoding LacZ-(CTG)_{400} or DMPK 11-15(CTG)_{300} cDNAs is shown. To quantitate the expression levels of expanded CUG repeat encoding transcripts, cDNAs (5 ng) from human myoblasts expressing LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} were subjected to Real-time PCR analysis in parallel. LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} cDNAs are present at approximately similar levels (Panels C, E & Table 6). Melting curves of LacZ-(CTG)_{400} or DMPK 11-15(CTG)_{300} PCR reactions are shown (Panels D & F). GAPDH was used as an internal control for RNA quality and the reverse transcriptase reaction (Ct values for GAPDH in LacZ-(CTG)_{400} and DMPK 11-15(CTG)_{300} samples was 19.8 in each case).

doi:10.1371/journal.pone.0003968.g008
Table 4. Aberrant RNA splicing results from the expression of expanded CTG repeats in myoblasts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alt. spliced exon number</th>
<th>n</th>
<th>DMPK 11-15(CTG)5</th>
<th>DMPK 11-15(CTG)300</th>
<th>% Exons splicing</th>
<th>GFP-DMPK 3′ UTR(CTG)5</th>
<th>GFP-DMPK 3′ UTR(CTG)300</th>
<th>LacZ-(CTG)5</th>
<th>LacZ-(CTG)300</th>
<th>Normal myoblasts</th>
<th>DM1 myoblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>11</td>
<td>3</td>
<td>52.4±2.3</td>
<td>62.1±1.7*</td>
<td>51.4±2.7</td>
<td>11.2±2.3*</td>
<td>43.7±3.5</td>
<td>46.9±2.9</td>
<td>46.7±2.7</td>
<td>6.2±3.1*</td>
<td></td>
</tr>
<tr>
<td>cTNT</td>
<td>5</td>
<td>3</td>
<td>30.5±1.9</td>
<td>83.9±2.6*</td>
<td>32.5±2.9</td>
<td>81.6±3.1*</td>
<td>27.3±1.8</td>
<td>30.1±2.3</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Asterisk (*) represents significant differences from the control (Student’s t-test; p < 0.05). IR, insulin receptor; cTNT, cardiac troponin T; ND, Not determined. doi:10.1371/journal.pone.0003968.t004

Table 5. Quantitation of levels of LacZ-(CUG)400 and DMPK 11-15(CUG)300 RNAs by RT-PCR.

<table>
<thead>
<tr>
<th>Plasmid constructs transfected into myoblasts</th>
<th>Relative expression (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ-(CTG)5</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>LacZ-(CTG)400</td>
<td>85.3±7.4#</td>
<td></td>
</tr>
<tr>
<td>DMPK 11-15(CTG)5</td>
<td>86.5±9.3</td>
<td></td>
</tr>
<tr>
<td>DMPK 11-15(CTG)300</td>
<td>76.4±7.5#</td>
<td>0.479</td>
</tr>
</tbody>
</table>

(#) represents pair wise comparison; p-value (Student’s t-test) denotes no significant difference in the expression levels of LacZ-(CTG)400 and DMPK 11-15(CTG)300 cDNAs.
doi:10.1371/journal.pone.0003968.t005

Table 6. Quantitation of LacZ-(CUG)400 and DMPK 11-15(CUG)300 RNAs by Real-time PCR.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>fmoles</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LacZ-(CTG)400</td>
<td>5.68×10^-4</td>
<td>0.06</td>
</tr>
<tr>
<td>DMPK 11-15(CTG)300</td>
<td>4.17×10^-4</td>
<td></td>
</tr>
</tbody>
</table>

p-value (Student’s t-test; p < 0.05) denotes no significant difference in the levels of LacZ-(CTG)400 and DMPK 11-15(CTG)300 cDNAs.
doi:10.1371/journal.pone.0003968.t006

Table 7. Telemetry measurements in conscious α-MHC-LacZ-(CTG)400 mice.

<table>
<thead>
<tr>
<th>LacZ (N = 5)</th>
<th>TG^high (N = 14)</th>
<th>TG^low (N = 9)</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL (ms)</td>
<td>99 ± 5</td>
<td>88 ± 9</td>
<td>ns (0.06)</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>609 ± 31</td>
<td>685 ± 71</td>
<td>668 ± 59</td>
</tr>
<tr>
<td>PR (ms)</td>
<td>35.4 ± 1.8*</td>
<td>31.9 ± 2.1*</td>
<td>33.4 ± 2.3</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>14.6 ± 0.7</td>
<td>14.0 ± 1.5</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>24.8 ± 1.5</td>
<td>23.4 ± 2.3</td>
<td>25.2 ± 2.9</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>25.0 ± 1.2</td>
<td>25.0 ± 2.7</td>
<td>26.8 ± 2.7</td>
</tr>
</tbody>
</table>

Parameter values represent mean ± standard deviation. Matching symbols (*) denote significant differences between groups in post hoc testing for that parameter; LacZ, α-MHC-LacZ-(CTG)400; TG^high, α-MHC-LacZ-(CTG)400; TG^low, α-MHC-LacZ-(CTG)300; SCL, sinus-cycle length; HR, heart rate; PR, atrial and A-V nodal conduction time; QRS, ventricular depolarization time; QT, surrogate of action potential duration; QTc, corrected surrogate of action potential duration; ms, milliseconds, bpm, beats per minute. doi:10.1371/journal.pone.0003968.t007

Table 8. ECG measurements in sedated α-MHC-LacZ-(CTG)400 mice.

<table>
<thead>
<tr>
<th>LacZ (N = 5)</th>
<th>TG^high (N = 14)</th>
<th>TG^low (N = 9)</th>
<th>One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL (ms)</td>
<td>147 ± 3</td>
<td>143 ± 19</td>
<td>142 ± 7</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>407 ± 8</td>
<td>427 ± 56</td>
<td>424 ± 20</td>
</tr>
<tr>
<td>PR (ms)</td>
<td>39.6 ± 2.8</td>
<td>37.3 ± 3.4</td>
<td>41.2 ± 5.5</td>
</tr>
<tr>
<td>QRS (ms)</td>
<td>13.9 ± 0.8</td>
<td>13.7 ± 2.5*</td>
<td>16.8 ± 1.8*</td>
</tr>
<tr>
<td>QT (ms)</td>
<td>24.5 ± 1.2#</td>
<td>22.6 ± 3.7*</td>
<td>30.0 ± 2.9*</td>
</tr>
<tr>
<td>QTc (ms)</td>
<td>20.2 ± 0.9#</td>
<td>19.1 ± 4.0*</td>
<td>25.2 ± 2.5*</td>
</tr>
</tbody>
</table>

Parameter values represent mean ± standard deviation; Matching symbols (#) denote significant differences between groups in post hoc testing for that parameter; LacZ, α-MHC-LacZ-(CTG)400; TG^high, α-MHC-LacZ-(CTG)400; TG^low, α-MHC-LacZ-(CTG)300; SCL, sinus-cycle length; HR, heart rate; PR, atrial and A-V nodal conduction time; QRS, ventricular depolarization time; QT, surrogate of action potential duration; QTc, corrected surrogate of action potential duration; ms, milliseconds, bpm, beats per minute.”
doi:10.1371/journal.pone.0003968.t008

MBNL1 and CUG-BP1 [25,26,32,33,39,45–49]. Specifically, expression of expanded CUG repeat sequences results in elevated steady-state CUG-BP1 protein levels, which plays a causative role in dysregulating splice site choice in a set of physiologically important RNAs implicated in DM1 both in cell culture and transgenic mouse experiments [25,33,39,45–49]. However, our previous results in DM1 myoblasts demonstrate that siRNA mediated reduction of CUG-BP1 levels does not correct aberrant IR splicing [46,47]. Thus these results demonstrate that although increased steady state levels of CUG-BP1 is sufficient to produce features of DM1, elevated CUG-BP1 levels may not be required for DM1 pathology to manifest, as reduction of CUG-BP1 levels does not correct the splice defects in DM1 myoblasts.
Inactivation of MBNL1 plays an important role in etiology of DM1 spliceopathy. Specifically, disruption of Mbnl1 in mice is sufficient to recapitulate key features of the disease [32]. We have shown that re-expression of MBNL1 is sufficient to rescue the IR splice defects in DM1 patient cells [46,47]. Consistent with our results, over expression of MBNL1 in transgenic mice expressing CTG tracts allows the rescue of both the splice defects and myotonia [50]. These data therefore demonstrate first, that functional inactivation of MBNL1 is sufficient to produce DM1 pathophysiology. Second, as MBNL1 mediated rescue serves to correct key features of DM1, inactivation of MBNL1 must be a necessary event that is required for the development of DM1 pathophysiology. The mechanism of MBNL1 inactivation is currently unknown. As previous studies have shown marked sequestration of MBNL1 in CUG foci both in skeletal muscle and heart cells [35,36], it has been hypothesized that MBNL1 depletion occurring as a consequence of aberrant sequestration, is a key mechanism that underlies the functional inactivation of MBNL1 in DM1.

As splice defects are not observed in α-MHC-LacZ-(CTG)400 mice, we studied the ability of the LacZ-(CTG)400 RNA to alter the stoichiometry or behavior of Mbnl1 and Cug-bp1. Similar, but relatively small amounts of endogenous Mbnl1, localize both in nuclear CUG foci (~8%) in DM1 myoblasts, where aberrant splicing is observed, and in cytoplasmic CUG-foci (~7%) in α-MHC-LacZ-(CTG)400 cardiomyocytes, where splice defects are not observed [Figure 4]. As experiments in Mbnl1+/− mice demonstrate that a ~50% decrease in Mbnl1 is insufficient to alter splice site choice [32], these data demonstrate that aggregation per se cannot be the sole mechanism that underlies MBNL1 inactivation in DM1 cells. Nonetheless, MBNL1 inactivation must be a key event in DM1 myoblasts as over expression of MBNL1 is sufficient to rescue the splice defects in these cells [46,47]. Thus as expression of LacZ-(CTG)400 RNAs is not sufficient to dysregulate RNA splicing, these RNAs must also by inference be unable to inactivate Mbnl1 function in vivo. The molecular basis of MBNL1 inactivation in DM1 myoblasts is currently unclear and is an important area of future investigation.

Significantly, steady state Cug-bp1 levels are elevated ~2.4 and ~1.5 fold in α-MHC-LacZ-(CTG)400TGsh and α-MHC-LacZ-(CTG)100TGsh mice respectively, at six months of age [Figure 5]. Experiments carried out by Lin and colleagues demonstrate that expression of ~250 CTG repeats located in the 3’UTR of α-actin gene does not result in elevated Cug-bp1 levels in mouse skeletal muscles [35]. These data demonstrate that CTG tracts, greater than 250 repeats, may be necessary to increase steady state Cug-bp1 levels, in adult mouse tissues. On inductive expression of very high levels of interrupted CTG tracts, Cug-bp1 levels are increased in the heart [24]. However as Cug-bp1 levels were not quantitated in this study, the exact increases in Cug-bp1 levels at time points at which splice defects manifest are unclear. Cug-bp1 over expression studies in mice demonstrate that a 4–6 fold elevation in Cug-bp1 levels [48] may be required to result in aberrant splicing. Therefore, in adult mouse tissues very high levels of Cug-bp1 may be necessary to elicit splice defects. If adult human tissues behave in a similar fashion, CUG-BP1 levels, which are high enough to dysregulate splicing, may result only in conjunction with the expression of very large CTG tracts. In this event, elevated CUG-BP1 levels may be more relevant in the etiology of congenital DM1 rather than adult onset DM1.

Our results show that CTG tracts are necessary but not sufficient to cause nuclear aggregation of CUG repeats. The precise RNA motifs required for nuclear aggregation of CUG repeats are currently unknown; however, the presence of such motifs must play a key role both in determining the ultimate location of the CUG repeat encoding RNAs. In this respect it is significant to note that both MBNL1 and hnRNP H have been shown to be required for nuclear focus formation [46,51]. Specifically, we have shown that siRNA mediated inactivation of MBNL1, which binds to the stem of the CUG hairpin, results in increased dispersion of nuclear foci in DM1 cells [46]. siRNA mediated reduction of hnRNP H, which binds to the base of the CUG hairpin, has also been demonstrated to allow transport of RNAs encoding expanded CUG repeats into the cytoplasm [51]. These results suggest that proteins that either bind to CUG hairpins or to key regions of the flanking sequence may facilitate hairpin stabilization and aggregate formation in the nucleus. As aggregation and transport into the cytoplasm may be two competing events within the nucleus, these data predict that small molecules, which serve to decrease the rate of aggregate formation in the nucleus, to allow transport of the toxic CUG RNAs into the cytoplasm, may be sufficient to greatly ameliorate DM1 symptoms in patients whose repeat tract sizes are not long enough to elicit large increases in CUG-BP1 levels. Thus small molecules that decrease the rate of either MBNL1 or hnRNP H binding to the mutant DMPK RNA may serve to increase its transport out of the nucleus. Such a cadre of drugs is attractive as they may prove to be less toxic, when compared to small molecules that abolish the interaction of these proteins with the mutant DMPK RNA, as their disruptive effect on MBNL1 or hnRNP H interactions with their normal target RNAs may be less severe.

**Materials and Methods**

**Cell culture and transfection**

Normal and DM1 myoblasts were a generous gift from Dr. Charles Thornton. DM1 fibroblasts were purchased from Coriell...
Institute for Medical Research, NJ, U.S.A. Normal and DM1 myoblast and fibroblasts cultures were immortalized by infection with the SV40 virus. Myoblast cultures and lines were maintained in SKGM medium (Lonza Inc., USA) containing 10% fetal bovine serum and fibroblast cultures and lines were maintained in MEM medium containing 20% fetal bovine serum.

**FISH analyses**

*In situ* fluorescence hybridization (FISH) analyses were carried out primarily as described by Tanca et al, 1995 [13] and Danisthong et al, 2005 [46]. Briefly, cardiomyocytes were prepared from mouse hearts using standard protocols [52] and plated immediately on chamber slides and fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature and stored in 70% ethanol at 4°C. Endogenous MBNL1 was detected using MBNL1 monoclonal antibody (MB1a) at a dilution of 1:200 [31]. Secondary antibody, anti-mouse IgG conjugated with FITC (Alexa Fluor 488, Molecular probes, USA) was used at a dilution 1:200. For FISH, a Cy3 conjugated (CAG)10 oligonucleotide probe (Operon, USA) was used to detect CUG repeat expansions as described by Tanca et al, 1995 [15]. To estimate the amount of MBNL1 present in the nucleus, the cytoplasm and the foci, the fluorescence signals (area x intensity) were measured using IP Lab software (Scanalytics Inc., USA). The percentage of MBNL1 present in the nucleus and cytoplasm of cardiomyocytes derived from α-MHC-LacZ mice and normal human myoblasts was calculated as: MBNL1nucleus = [MBNL1whole cell x MBNL1nucleus x 100]; MBNL1cytoplasm = [MBNL1whole cell x MBNL1cytoplasm x 100]. For cardiomyocytes derived from α-MHC-LacZ-(CTG)400 mice the percentage of MBNL1 in nucleus, cytoplasm and foci was calculated as: MBNL1nucleus = [MBNL1whole cell x MBNL1nuclear foci x 100]; MBNL1cytoplasm = [MBNL1whole cell x MBNL1cytoplasmic foci x 100]; MBNL1nuclear foci = MBNL1nucleus - MBNL1cytoplasmic foci; MBNL1cytoplasmic foci = MBNL1cytoplasm - MBNL1nuclear foci;

**Generation of α-MHC-LacZ-(CTG)400 and α-MHC-LacZ mice**

(CTG)400 tracts were cloned into an SfiI site located within a linker sequence (TGGCCACCGGGCGCATTTAATGCGCATTAGGGCC; SfiI sites are underlined) that was inserted into the termination codon of the β-galactosidase gene and the bovine growth hormone polyadenylation (BGH-PolyA) sequence. The α-myosin heavy chain (α-MHC) promoter was used to drive expression of the transgene [28]. Both the α-MHC-LacZ- (CTG)400 cassette and control α-MHC-LacZ cassette, which did not encode an expanded CTG tract were injected into fertilized C57BL/6J mouse eggs to generate two lines and two control α-MHC-LacZ lines (α-MHC-LacZ-(CTG)0). The transgenic mice were genotyped by PCR using β-galactosidase specific primers (forward 5'-ATGATGATCCCGTCGTTTT ACAAC-3' and reverse 5'-TCAATACG66CGGCTGC-3').

**Southern and Northern blot analyses**

For Southern blot analyses, genomic DNA from transgenic mice and extracts were prepared by homogenization with the extraction buffer [25 mM Tris-HCl pH 7.6, 400 mM NaCl, 0.5% NP40, 5 mM EDTA, 25% Sucrose, 2 mM PMSF and Protease inhibitor (Sigma Inc., Catalog # P-8340)]. Tissue extracts were incubated on ice for 1 hour and then centrifuged for 20 min at 20,000g. Equal amounts of protein (either 6 μg or 10 μg) were resolved by SDS-PAGE and transferred to Hybond P membranes (Amersham Bioscience Inc., USA). After blocking with 5% skim milk in 0.1% Tween 20 in PBS, the membranes were incubated with primary antibodies for 2 hrs at room temperature or overnight at 4°C. The membranes were washed with 0.1% Tween 20 in PBS and subsequently incubated with the corresponding secondary antibodies conjugated with HRP. Signals were detected by using the ECL plus detection reagents (Amersham Bioscience Inc., USA) according to the manufacturer’s protocol. The primary antibodies were CUG-BP1 [3B1 monoclonal antibody (200 μg/ml), Santa Cruz Inc. (catalog # sc-20003), 1:4000], and MBNL1 monoclonal antibody (MB1a, 1:3000) [31] to detect the Cug-bp1 and Mbn1l. To control for protein quality and loading the membranes were probed with goat anti-GAPDH (V-18 polyclonal antibodies (200 μg/ml), Santa Cruz Inc. (catalog # sc-20357)) at a dilution of 1:3000. The secondary antibody dilutions were 1:8000 for goat anti-mouse IgG-HRP ([1 mg/ml], Sigma Inc. (catalog # A2304)), and 1:5000 for donkey anti-goat IgG-HRP ([400 μg/ml], Santa Cruz Inc. (catalog # sc-2056)). The relative band intensities were measured by densitometry analyses using Gene Tool (Syngene Inc., USA). To ensure that the signals were not saturated, prior standardizations were carried out as described in Paul et al, 2006 [47].

Sub-cellular fractions (cytoplasmic and nuclear) from mouse heart tissue was prepared using methods primarily described by Charlet et al, 2002 [49] with some modifications. Heart tissues were collected from transgenic mice and homogenized with cold lysis buffer (25 mM Tris-HCl [pH 7.6], 10 mM NaCl, 1.0 mM EDTA, 2.0 mM MgCl2, 0.5 mM DTT, and protease inhibitor). The soluble fraction was passed through a 26-gauge needle eight times using a 1 ml syringe. Nuclear: cytoplasmic separations were carried out by centrifugation twice at 600g for 5 min. The cytoplasmic extract was prepared by centrifuging the supernatant fraction for 20 min at 20,000g. The pellet from the nuclear: cytoplasmic separations was suspended in nuclear extraction buffer (25 mM Tris-HCl [pH 7.6], 600 mM NaCl, 0.05% NP-40, 1.0 mM EDTA, 2.0 mM MgCl2, 0.5 mM DTT, and protease inhibitor) and centrifuged for 20 min at 20,000g. The resulting supernatant was denoted as a nuclear extract. Equal amounts of protein (10 μg) from the nuclear and cytoplasmic fractions were resolved by SDS-PAGE and transferred to Hybond P membranes. The membranes were probed with TBP (TATA binding protein) monoclonal antibody ([1.99 mg/ml], Abcam Inc. (catalog # ab818), 1:5000), which was used as a nuclear marker and goat anti-GAPDH (V-18 polyclonal antibodies (200 μg/ml),...
RT-PCR analyses of RNA isolated from transgenic mice

Total RNA was isolated from transgenic mouse hearts using Trizole (Invitrogen, USA) according to the manufacturer’s protocol. cDNA was synthesized from 3 μg of total RNA using the cDNA synthesis kit (Amersham Bioscience Inc., USA) and PCR was carried out using 150 ng of the synthesized cDNA. PCR amplification was carried out for 25 and 30 cycles for each gene. The sequences of the primers used for the amplification of Tmrt2, Alp, Zasp, and m-Tn are: Tmrt2 (forward 5’-GGGAGGAGGTGTTTGAGAAGG-3’, reverse 5’-GGGGAGGTGTTTTTCTT-3’), Alp (forward 5’-AGCTGTGTCCT-3’, reverse 5’-GATCTGGCTCCTCA-3’), Zasp (forward 5’-GGAGAGTGAAGCTGTGATTG-3’, reverse 5’-TGGTGACAGTGGTTGCTT-3’), and m-Tn (forward 5’-GTTGATCGCTCAGGAGACCGA-3’, reverse 5’-CCACCACAGGACCATGGTTTCC-3’). To ensure that the signals were not saturated, prior standardization experiments were carried out as described in Paul et al, 2006 [47]. Signals were normalized to the expression of GAPDH and quantitated as percent relative expression. Real-time PCR was carried out to quantitate the expression levels of LacZ-(CUG)400 and DMPK 11-15(CUG)300 transcripts. The primers used in Real-time PCR are: LacZ-(CUG)400 constructs (forward 5’-AGATAGATATGCCCGTGTTTAC-3’, reverse 5’-CGGCTATTACGGGTCCAACTTG-3’), DMPK 11-15(CUG)300 constructs (forward 5’-GAATGACATGACCCGACATCC-3’, reverse 5’-ATTATGATCGGTATCTCGATG-3’), and GAPDH (forward 5’-CCACCACGGGAAATTTCCATG-3’, reverse 5’-TATGGGATTTCCATGATGAC-3’). The PCR reaction mixture contained SYBR Green PCR Master Mix (Bio Rad, USA) and 0.5 pmol primers. PCR amplification was carried out for 45 cycles: denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min.

Electrocardiography Studies

A total of 35 adult C57BL/6j strain mice were studied. The mean age was 27±8 weeks and mean weight was 30±5 grams of all mice. Mice were anesthetized with intraperitoneal pentobarbital (0.033 mg/gm each). A 6-lead surface ECG was obtained with 25 gauge electrodes placed subcutaneously in each limb. Mean sinus-cycle length (SCL), heart rate (HR), PR, QRS, and QT intervals were measured for each animal as described by Berul et al, 1996 [33]. A rate corrected QT interval (QTc) was calculated using a murine formula [54].

Ambulatory Electrocardiogram Telemetry

Radiofrequency transmitters (DataSciences International, St. Paul, MN, USA) were implanted into a subcutaneous pocket with leads secured under the upper right and left limbs to record lead I ECG. After a 48-hours recovery period, ECGs were recorded continuously for 10 minutes. All baseline telemetry measurements for SCL, PR, QRS and QT intervals were made for three consecutive cardiac cycles by an experienced observer blinded to the genotypes of the mice.

Exercise Tolerance Test

Animals with implanted transmitters were exercised on a multilead graded treadmill machine (Exer-6M, Columbus Instruments, Columbus, OH, USA). Mice were encouraged to run for 30 minutes at a constant speed of 12.5 m/min at a slope of 15 degrees. Telemetry ECG recordings and measurements were made at rest, after 10 and 20 minutes of exercise, just before.
termination of exercise, and at recovery. During exercise and recovery, telemetric ECG recordings were examined for conduction abnormalities and the presence of inducible arrhythmias.

Statistical Analysis
Statistical analyses were performed with SPSS software version 11.5 for Windows. Continuous variables, such as ECG intervals, cardiac conduction properties and echocardiography measurements were compared for genotypes using an analysis of variances (ANOVA) test followed by post-hoc analysis. Pearson chi-square tests were performed for categorical data. Values are presented as the mean ± 1 standard error of mean (SEM). The Student t-test was used to analyze data derived from RNA splicing, expression analyses and the examination of MBNL1 cellular localization. Statistical significance was established with a p value of <0.05.

Supporting Information
Figure S1 Images of DM1 cells containing both nuclear and cytoplasmic foci. Panels A–E: Nuclear DAPI stains of normal and DM1 myoblast and fibroblast cultures and SV40 transformed lines. Panels A–E: Nuclear DAPI stains of normal and DM1 myoblast and fibroblast cultures and SV40 transformed lines. The confocal microscopy facility in the Doheney Eye Institute at the University of Southern California was used for FISH analyses.

Acknowledgments
We thank Dr. Maurice Swanson for the Mbnl1−/− mice and Dr. Charles Thornton for normal and DM1 myoblast cultures. The confocal microscopy facility in the Doheney Eye Institute at the University of Southern California was used for FISH analyses.

Author Contributions
Conceived and designed the experiments: WD SP SR. Performed the experiments: WD CW PS SP AC DB MS. Analyzed the data: WD CW SP SR. Contributed reagents/materials/analysis tools: WD SP IH GM CB SR. Wrote the paper: WD SP SR.

References

In DM1 cells, ~70% and ~30% of all sampled cells [number of cells counted in each case are shown in Figure 1; Table 1 of the main text] showed nuclear foci or both nuclear and cytoplasmic, respectively.

Found at: doi:10.1371/journal.pone.0003968.s001 (2.66 MB TIF)

Figure S2 MBNL1 monoclonal antibody (MB1a) specifically detects Mbnl1 in mouse cardiomyocytes. Nuclear DAPI stains of cardiomyocytes derived from wild type, and Mbnl1−/− mice (a gift from Dr. Swanson MS) are shown in a and c. Distribution of endogenous Mbnl1 was visualized as a green signal in wild type cardiomyocytes (b) using anti-MBNL1 (MB1a) monoclonal antibody and a secondary antibody (anti-mouse IgG) conjugated with FITC. Mbnl1 is not detected in Mbnl1−/− cardiomyocytes (d).

Found at: doi:10.1371/journal.pone.0003968.s002 (3.93 MB TIF)