Sustainable recovery of MBNL activity in autoregulatory feedback loop in myotonic dystrophy

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Muscleblind-like proteins (MBNLs) are RNA-binding proteins essential for the developmental regulation of various processes including alternative splicing. Their activity is misregulated in myotonic dystrophy type 1 (DM1), an incurable genetic, neuromuscular disorder caused by uncontrolled expansion of CTG repeats. Mutant RNAs containing hundreds or thousands of repeats efficiently sequester MBNL proteins. As a consequence, global alternative splicing abnormalities are induced. Importantly, the size of expansion differs significantly not only between patients but also between different parts of the same muscle as a consequence of somatic expansion. One of the potential therapeutic strategies in DM is overexpression of MBNLs. However, gene therapy tools might induce excessive activity of MBNLs, what in turn might change the metabolism of many RNAs. To overcome these limitations, we designed an autoregulated MBNL1 overexpression system. The genetic construct contains an MBNL1-coding sequence separated by the fragment of ATP2A1 pre-mRNA with an MBNL1-sensitive alternative exon containing stop codon in the reading frame of MBNL1. Inclusion of this exon leads to the arrangement of an inactive form of the protein, but exclusion gives rise to fully active MBNL1. This approach enables the autoregulation of the amount of overexpressed MBNL1 with high dynamic range which ensures a homogeneous level of this protein in cells treated with the genetic construct. We demonstrated beneficial effects of an autoregulated construct on alternative splicing patterns in DM1 models and cells derived from patients with DM1.

INTRODUCTION

Three Muscleblind-like genes (MBNLs) encode for RNA-binding proteins essential for regulation of various processes of RNA metabolism including alternative splicing, polyadenylation, RNA localization, and stability.1-3 MBNL1 is mainly expressed in skeletal muscles, MBNL2 in brain, and MBNL3 in placenta and during muscle cell differentiation, where they regulate hundreds of alternative splicing events leading to the expression of adult-specific mRNA isoforms.1-6 All family members bind to RNA via four zinc fingers (ZFs) organized in two tandems connected by a long linker. MBNLs recognize their targets through closely organized multiple 5'-YGCY-3' sequence motifs, where Y represents a pyrimidine.7 The location of the MBNL-bind-
Hence, the sequestration of MBNL proteins is highly heterogeneous in both DM forms, even in the same tissue of the same patient.

So far, there are no cures for these diseases; however, a few therapeutic approaches were tested in cell and animal models of DM, and some of them demonstrated rescue of disease phenotype. The therapeutic strategies for DM1 may be grouped into three main categories: (1) induction of degradation of mutant RNA containing CUG<sup>RPM</sup>, (2) the release of MBNLs from pathogenic sequestration, and (3) the increase of MBNL expression. The second category involves the application of CUG<sup>RPM</sup>-specific small molecules or antisense oligonucleotides (ASOs), which can inhibit the formation of the pathogenic RNA-MBNL complex and lead to the improvement of alternative splicing defects.25–28

The third mentioned category involves either manipulation of an endogenous pool of MBNLs, e.g., the use of non-steroidal anti-inflammatory drugs that modify activity of MBNL1 promoter,29 or antagoniRs targeting mir-23b or mir-218 to increase translation from MBNL1 and MBNL2 mRNAs,30 or the application of gene therapy tools for MBNL overexpression.

Proof of concept for gene therapy was described for the DM1 mouse model, HSA-LR, expressing a transgene with 220 CTG repeats in 3' UTR of human skeletal actin gene (HSA). The MBNL1-encoding transgene was delivered to the HSA-LR mouse via transduction with adenov-associated virus (AAV).30 Twenty-three weeks after intramuscular AAV injection, the MBNL1 overexpression rescued the splicing defects of many MBNL-sensitive genes. At the same time, muscle hyperexcitability was missing. Otherwise, the normal structure of myofibers was not recovered, suggesting that the overexpression was insufficient to rescue this phenotype.30 More recently, it was shown that truncated MBNL1 overexpressed from the AAV vector, which preserved both ZFs tandems but is deprived of the C-terminal domain, binds to the CUG<sup>RPM</sup> with high affinity, leading to the release endogenous MBNL proteins from sequestration and then rescue of disease phenotypes in the DM1 mouse model.31 In other research, it was shown that the cross of HSA-LR with the mouse model with multisystemic overexpression of MBNL1 or overexpression restricted just to the skeletal muscles (MBNL1-OE) showed the rescue of DM-like defects, including the decrease of percentage of fibers with central nuclei reflecting myopathy changes. On the other hand, the long-term, multisystemic overexpression of MBNL1 in wild-type mice and another DM1 mouse model, DM200<sup>0</sup>, which utilizes the doxycycline-dependent promoter that controls the expression of a transgene with 200 CTG repeats in the 3' UTR of the DMPK gene, led to reduced body weight and increased mortality.32,33 Moreover, intramuscular injection of AAV encoding the full length of MBNL1 to the wild-type (WT) mice induced formation of muscle fibers with internal nuclei, which indicates muscle damage.31 These results suggest that there are some limitations in therapeutic strategies against DM based on the uncontrolled overexpression of MBNLs, especially considering the significant variability in the size and expression level of CUG<sup>RPM</sup> in different tissues of patients with DM.

To overcome the limitations caused by heterogeneity of CTG/CCTG repeat expansions and, consequently, different levels of MBNL insufficiency in different cells/myoﬁbbers, we designed and tested the autoregulated MBNL1 overexpression constructs, which enable the significant production of MBNL1 only if its level in the cell is too low and potentially can be controlled by heterogeneity of the CUG<sup>RPM</sup> load. We demonstrated that the level of the protein assembling from the construct is homogeneous from cell to cell, is controlled by a pool of available MBNLs, and has therapeutic potential to correct the alternative splicing abnormalities in cellular models of DM1. Considering DM1 and DM2 acquire the same mechanism of MBNL sequestration and, in consequence, share similar missplicing events, the potential therapeutics may be suitable for both.34

RESULTS

Constructs for autoregulated overexpression of MBNL1auto

For the autoregulated overexpression of MBNL1, we designed the hybrid genetic construct MB22#1, which contains an MBNL1-encoding sequence separated by a fragment of the ATP2A1 gene containing MBNL-sensitive alternative exon 22 (ex22) and neighboring introns (Figures 1A and S1). We decided to choose ex22 because of its high sensitivity to MBNL regulation. In skeletal muscles, the isoform with the inclusion of ex22 predominates, but in affected muscles of patients with DM1, different levels of ex22 exclusion are observed (Figure 1B), which is correlated with disease severity.35 The sequence of the ATP2A1 gene fragment was incorporated between ex2 and ex3 of MBNL1 so as to not disturb the structure of any of the ZF tandems. Alternative ex22 is positively regulated by all MBNL paralogs.36 We hypothesized that the inclusion of ex22 may lead to the production of a truncated, inactive form of the protein because of the presence of an in-frame stop codon (Figure 1A). Therefore, in cells with low levels of MBNLs, ex22 can be excluded during pre-mRNA maturation, and the fully active form of MBNL1 protein (MBNL1auto) can be assembled. We also designed two other constructs by replacing native MBNL-binding sequences in intron 22 with sequences showing different sensitivities to MBNLs.37 First, MB22-<del>del has a deletion of the native MBNL-binding site of ATP2A1 (Figure 1A). Moreover, at the C terminus of the MBNL1auto sequence, either FLAG tag or GFP tag was added. We hypothesized that these constructs allow the adjustment of different amounts of MBNL1auto due to different efficiency of ex22 inclusion.

To test this hypothesis, we co-transfected COS7 cells with one of three generated MB22 constructs containing different MBNL-sensitive elements and either MBNL1-GFP (without [w/o] autoregulatory cassette) or as a control GFP expressing vector. The RT-PCR base splicing assay showed that the percentage of mRNA isoforms with the exclusion of ex22 depends on the presence of an MBNL-sensitive RNA regulatory element (MB22#1, -#2, or -del) and the level of the MBNL pool in cells. We decided to measure the ex22 exclusion, as this isoform can produce functional MBNL1auto. As expected, the exclusion rate of ex22 was higher for MB22#1 and MB22#2 constructs in cells with a basal pool of endogenous MBNLs and significantly

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decreased with MBNL1-GFP overexpression (Figure 1C). Unexpectedly, the level of mRNA with excluded ex22 from the control MB22-del construct is very low because the isoform with retention of both introns predominates.

Next, we analyzed the level of MBNL1auto in cells transfected with one of three autoregulated MB22 constructs (Figure 1D, left graph) or co-transfected with MB22s and non-autoregulated MBNL1-GFP (Figure 1D, right graph) using western blot. A higher amount of protein was produced in cells treated with MB22#1. On the other hand, a similar amount of MBNL1auto protein was detected in cells co-transfected with either MB22#1 or MB22#2 and MBNL1-GFP. These results are consistent with a RT-PCR-base splicing assay showing the percentage of mRNA isoforms with exclusion of ex22 (Figure 1C). The highest ex22 exclusion was in cells treated with MB22#1, suggesting the highest potential to produce the full length of MBNL1auto. Furthermore, we evaluated the total pool of MBNL1 after MB22 over-expression. The western blot analysis showed about a 2-times increase of the MBNL1 pool compared with the endogenous level of this protein (Figure S2). MBNL1auto was not detected in cells transfected with MB22-del (Figure 1D), which corroborated the results of mRNA splicing analysis (Figure 1C).

All these experiments demonstrate that autoregulated MB22 constructs are capable of overexpressing different levels of MBNL1auto in cells with different pools of MBNL proteins.

**The effect of a pool of MBNLs on production of MBNL1auto from the autoregulated MB22 construct**

By design, the amount of produced MBNL1auto should also differ in cells with different endogenous MBNL pools. Therefore, we over-expressed MB22#1 in three cell lines with different levels of MBNL pools (total of MBNL1, -2, and -3): in the COS7 line and the HEK293 line, with relatively high and low endogenous pools of MBNLs,
respectively, and mouse embryonic fibroblasts with full knockout of Mbnl1 and Mbnl2 (MEF-1&2KO). These cells were co-transfected with either MBNL1-GFP to increase the pool of MBNLs or GFP used as a negative control (Figure 2). Overexpressed MBNL1-GFP, similar to endogenous MBNLs, can bind to the regulatory element within the intron of MB22#1 pre-mRNA, leading to ex22 inclusion and premature termination of translation (ex22 contains a stop codon), which decreases the level of MBNL1auto. Moreover, COS7 cells were also transfected with small interfering RNA (siRNA) against MBNL1 (siMBNL1), targeting the sequence within the 3’ UTR to not disturb expression of the MB22 construct. As expected, siMBNL1 treatment significantly increased the level of MBNL1auto by about 2.5 times. On the other hand, the overexpression of MBNL1-GFP has a negative effect on the level of protein from the MB22#1 construct in each tested cell line (Figure 2), leading to an about 3-fold decrease of MBNL1auto in HEK293 and MEF-1&2KO cells. Taken together, these results indicate that production of MBNL1auto strongly depends on the changing level of the MBNL pool in a broad range of MBNL concentrations.

The level of MBNL1auto is homogeneous in cells

To achieve insight into the ability of the MBNL1auto protein to autoregulate, we co-transfected COS7 with different amounts of construct encoding either MBNL1auto or MBNL1-GFP (100–1,000 ng) and minigenes containing MBNL-sensitive alternative exons (MBNL1 ex5, Nfix ex7, Atp2a1 ex22) (Figures 3A and 3B). This approach gave us the comparison of MBNL1 proteins that came from either autoregulated or non-autoregulated constructs on depth and dynamics of alternative splicing regulation. Results of RT-PCR assay showed that the percentage of inclusion of ex22 from MB22#1 is indeed not sensitive to the amount of transfected construct (Figure 3A). Moreover, increasing amounts of transfected MB22#1 did not significantly differ in the regulation of three other alternative exons (Figure 3B). In contrast, MBNL1-GFP showed concentration-dependent splicing regulation, reaching saturation for the highest amount of overexpression construct (Figure 3B). A different pattern of ex22 inclusion from Atp2a1 minigene does not demonstrate better activity of MBNL1-GFP than the MBNL1auto, as this minigene responds to low levels of MBNL1 overexpression. Collectively, these results suggest that expression of MBNL1auto can reach a certain maximal level independently from amount of MB22 genetic construct delivered into cells.

To directly measure the diversity of MBNL1auto-GFP production in MB22#2-GFP-transfected COS7 cells, we utilized the flow cytometry approach (Figure S3A). Quantification on resolution of a single cell performed in two independent experiments revealed that the level of MBNL1auto-GFP is more homogeneous (4-times difference in a GFP signal between the 25th and 75th percentiles of analyzed cells) than the level of MBNL1-GFP (13-times difference) which was used as a negative control (Figures 3C and S3B). We confirmed an equal level of MBNL1auto-GFP using confocal microscopy (Figure 3D). Taken together, these findings revealed that MBNL1auto-GFP shows homogeneous expression in transfected cells.

MBNL1auto corrects alternative splicing abnormalities in DM1 cell models

To investigate therapeutic potential of MB22, we first studied if the MBNL1auto-GFP protein can bind to the CUG repeats. COS7 cells were co-transfected with a MB22#2 construct expressing MBNL1auto fused with GFP and either a construct expressing a mutant DMPK mRNA fragment containing ex11-ex15 with 960 interrupted CUG repeats in the 3’ UTR (CUG960) or a normal DMPK fragment without
repeats (CUG0). In cells with CUG960, the fluorescent signal from MBNL1auto-GFP protein is located mostly in the ribonuclear foci. In contrast, diffuse distribution and, again, homogeneous GFP signals between cells were observed in a control experiment in cells with CUG0 (Figure 4A). Moreover, we also performed RNA fluorescence in situ hybridization (FISH), which also confirmed co-localization of MBNL1auto-GFP with CUGexp, using a probe labeled with Cy-3, which detects CUGexp (Figure S4). These experiments showed that MBNL1auto, by binding to CUGexp in cell nuclei, can replace endogenous MBNLs from sequestration.

Therefore, next we looked at the potential of MBNL1auto on the correction of MBNL-sensitive alternative splicing in two DM1 cell models. In the first, COS7 cells were co-transfected with MB22#1 and mutant or normal DMPK-expressing constructs (CUG960 or CUG0) together with two MBNL-sensitive minigenes (Nfix ex7, Atp2a1 ex22). RT-PCR analyses showed that the presence of MBNL1auto leads to the partial, but significant, rescue of all tested DM1-specific alternative splicing events (Figure 4B). To further support these results, we also utilized other cellular models with siRNA-induced insufficiency of MBNLs (siMBNL1; as described above). Cells with silenced endogenous MBNL1 were co-transfected with MB22#1 and the same splicing minigenes (Nfix ex7, Atp2a1 ex22). We confirmed that siMBNL1 efficiently knocked down MBNL1 (Figure S5) and significantly increased the level of MBNL1auto (Figure 2). Then, using splicing-specific RT-PCR assays, we demonstrated that MBNL1auto rescued the pathogenic missplicing triggered by MBNL1 deficiency (Figure 4C).

**Lentiviral-based production of MBNL1auto corrects splicing abnormalities in cells derived from patients with DM1**

The promising results obtained in the two DM models described above encouraged us to assess the therapeutic potential of MB22 in two different cells derived from patients with DM1 that expressed DMPK transcripts with very long CUGexp (DM1-1 and DM1-2). Previously, we showed that these cells manifest DM1-specific molecular phenotypes like the formation of CUGexp nuclear foci, sequestration of MBNLs, and missplicing of several transcripts.25,39 In this study, we utilized lentiviral vectors encoding MBNL1auto-GFP from autoregulatory MB22#2-GFP or GFP used as a negative control. The fusion with GFP was used to monitor the efficacy of transduction. The fibroblast from a healthy individual was used as a control cell line (non-DM). Twelve days after transduction, we checked the expression level of the DMPK gene to assess the potential effect of the production of...
MBNL1auto. The quantitative real-time RT-PCR analysis showed no differences in the steady-state level of DMPK mRNA in samples treated with different vectors (Figure 5A). Next, we evaluated alternative splicing changes of eight mRNAs that are known to be MBNL sensitive: INSR ex11, FLNB ex31, MY05A ex33, MBNL2 ex5, MBNL2 ex7, MBNL1 ex1, NCOR2 ex19, and PHKA1 ex19. All of them showed significant correction in both DM1 cell lines (Figures 5B and S6). Importantly, no significant splicing changes were observed in the non-DM1 cell line treated with MB22#2-GFP lentivirus (the only exception is a small change of ex19 inclusion in PHKA1). Cumulatively, all these data strongly suggest that the MBNL1auto protein, whose production is autoregulated on the level of alternative splicing of its mRNA, has the ability to rescue alternative splicing alterations in different DM1 models.

DISCUSSION

Gene therapy is promising strategy for many incurable diseases. It involves the delivery of new genetic material to patient cells to prevent or slow down development of a particular disease. This kind of treatment enables the efficient cure for some monogenic disorders, including spinal muscular atrophy (Zolgensma). Overexpression can rescue proper protein function but, when uncontrolled, may also trigger undesirable effects. Therefore, an important limitation to overcome in the gene therapy approach is to adjust the level of overexpressed protein to prevent toxicity but enable the therapeutic effect.

During different stages of tissue differentiation and development, the level of the MBNL pool is crucial for proper alternative splicing regulation. Expression of MBNL1 and MBNL2 increases during both embryonic and postnatal stages of development, with the highest level of MBNL1 in adult muscles and the highest MBNL2 in the adult brain. All three MBNL paralogs regulate the same splicing events; however, among them, fast and slow responders exist. Some alternative exons respond to low and some to high levels of MBNLs, and the depth of exon inclusion/exclusion could be regulated in a wide range of concentration of these proteins. Therefore, fine-tuning of the MBNL pool is important for proper function of different tissues.

In DM, the activity of all MBNL paralogs is significantly lower due to sequestration of these proteins on toxic CUG<sup>exp</sup> or CCUG<sup>exp</sup>, and the increase of MBNLs is considered a potential therapeutic strategy, including the application of gene therapy tools. It was shown that AAV-based production of the MBNL1 protein or its truncated variant rescued muscle hyperexcitability and splicing defects of many MBNL-sensitive genes. Moreover, expression of MBNL1 from a transgene in knockin mouse models also improved the DM-specific phenotype. On the other hand, overexpression of full-length MBNL1 induced by intramuscular injection of AAV vector in WT mice showed significant muscle damage. Also, transgenic mice overexpressing MBNL1 from a transgene demonstrated significantly decreased body weight, grip strength, run distance, and heart failure compared with WT littermates or even reduced survival. Moreover, uncontrolled and unbalanced overexpression might lead to the excessive activity of MBNLs in some treated muscle fibers or other tissues and, consequently, change the metabolism of many RNA.
Therefore, in this study we decided to develop the self-regulating MB22 overexpression construct for fine-tuning MBNL1auto production, which depends on the endogenous MBNL pool available in the cell. In designing MBNL1auto, we selected the sequence of the 41 kDa isoform of MBNL1. This isoform is deprived of alternative ex5, which is responsible for nuclear localization of the protein, but contains alternative ex7, which increases splicing activity of the protein.\(^4,48,49\) Importantly, this isoform is localized in both the nucleus and cytoplasm and can regulate not only alternative splicing but also other RNA metabolism stages\(^3\) and is one of major isoforms present in skeletal and cardiac muscles in adults.\(^46\) It was already demonstrated that the MBNL1 isoform with ex7 can efficiently bind to CUG\(^{exp}\) and CCUG\(^{exp}\).\(^46\)

Previous studies of ours and others showed that activity of MBNLs can be autoregulated on the level of alternative splicing.\(^4,50\) Therefore, we decided to design the autoregulatory MB22 construct, whose expression is sensitive to the MBNL pool, by adding the intron/ex22/intron sequence from ATP2A1 between the sequence encoding MBNL1 (Figure 1A). MBNL-dependent alternative splicing of pre-mRNA from this

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**Figure 5.** Correction of pathogenic missplicing in DM1 cells treated with MB22 lentiviruses

(A) Results of quantitative real-time RT-PCR analysis showing relative expression of DMPK (normalized to GAPDH) in three different cell lines: fibroblasts derived from healthy individual (non-DM) and two patients with DM1 (DM1-1 and DM1-2) treated with either control (CTRL) lentiviral vector or lentivirus containing the MB22#2-GFP sequence 12 days from cell transduction. (B) Results of RT-PCR-based analyses of alternative splicing changes in cells described in (A). Changes in the inclusion of positively (top panel) and negatively (bottom panel) regulated MBNL-dependent alternative exons are shown for six transcripts affected in DM1. Splicing changes are expressed as PSI. Bars represent average from 3 to 4 independent experiments (dots); unpaired Student’s t test; ‘*’p < 0.05; ‘**’p < 0.01; ‘***’p < 0.001; ns, non-significant.
construct is regulated by the efficiency of the inclusion of alternative ex22 containing an in-frame stop codon, and the inclusion of this exon leads to the production of a non-functional short protein.

As proposed in this study, an autoregulated MB22 construct enables the restricted expression of MBNL1auto (Figure 2), which is sufficient for the rescue of DM-specific alternative splicing defects in cellular models with insufficiency of MBNLs induced by either silencing of MBNL1 or overexpression of toxic CUG<sup>esp</sup> (Figure 4).

In muscles of patients with DM1 and DM2, the size of CTG or CCTG repeat expansion differs significantly between patients but also between different parts of the same muscle of the same individual, due to somatic instability of repeat tract. Hence, the sequestration of MBNL proteins is highly heterogeneous. Therefore, we hypothesized that unequal sequestration of the MBNL pool can be buffered by self-regulated overexpression of MBNL1auto. This was partially demonstrated in experiments with transfection-based and uncontrolled delivery of the MB22-GFP construct to model cells (Figure 3). Using conventional overexpression systems, we can expect high heterogeneity in the number of transcripts and proteins generated from the transgene, e.g., as a result of different numbers of copies of plasmid per single cell. Quantitative flow cytometry experiments and microscopic analyses (Figures 3C, S3B, and S3D), as well as monitoring of MBNL-sensitive splicing patterns (Figure 3B), showed that the production of MBNL1auto is homogeneous from cell to cell even after imbalanced delivery of the MB22 genetic construct. Importantly, the buffering level of MBNL1auto leads to significant correction of splicing abnormalities in cellular models of DM1 (Figures 4 and 5).

The therapeutic effect would be caused partially by binding of MBNL1auto to CUG<sup>esp</sup> and replacement of the endogenous pool of MBNLs from sequestration (Figures 4A and S4) and partially by the splicing activity of MBNL1auto itself. Therefore, in further studies, the MBNL-sensitive RNA regulatory element in MB22 can be easily replaced, giving the opportunity to better adjust the buffering level of the MBNL1auto. The regulatory element with lower affinity to MBNL proteins can be used to reduce sensitivity of ex22 inclusion and, consequently, increase the levels of arranged protein, keeping still the control on excess activity of MBNLs. Moreover, the use of a well-selected tissue-specific promoter and a proper delivery system could be adjusted.

Taken together, our results highlight the utility of autoregulated overexpression of MBNL1auto as a potential therapeutic tool in DM1, DM2, and other diseases in which MBNL proteins are sequestrated or insufficient.

**MATERIALS AND METHODS**

**Genetic constructs**

The MB22 construct was prepared by PCR amplification of an ATP2A1 gene fragment from human genomic DNA (for primers, see Table S1) and cloning into the previously described pEGFP-C1-MBNL1-41 vector for MBNL1 overexpression with removed GFP sequence. The MBNL1 sequence contains alternative ex7 and is deprived of alternative ex5, which contains a nuclear localization signal (Figure S1). The MB22-del and MB22#2 constructs were obtained by deletion of the fragment with MBNL-binding motifs and the replacement of the WT fragment with the 4xUGCU sequence, respectively. The MB22#2-GFP was prepared by an amplified EGFP sequence from pEGFP-C1 (CloneTech) and fusion to the C-terminal end of MBNL1. All the above constructs were prepared using the InFusion Cloning method. The desired fragments were amplified using CloneAmp HiFi PCR Premix (TakaraBio) and cloned using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) following the manufacturer’s instructions. The pEGFP-C1-MBNL1-41 vector used in this study (referred to as MBNL1-GFP overexpression vector) was previously described. The DT960 (CUG<sub>960</sub>) and DT0 (CUG<sub>0</sub>) vectors were a gift (Prof. Thomas Cooper, Baylor College of Medicine) and were already described. The pEGFP-C1 vector (CloneTech) was used as a control and is referred to as GFP. The Atp2a1 ex22<sup>52</sup> and Nfix ex7 minigenes, gifts from Prof. Manuel Ares, University of California, Santa Cruz, were described earlier. The MBNL1 ex5 minigene is described in the manuscript (Taylor K. et al., unpublished data). The sequences of primers used in cloning are listed in Table S1. The lentiviral vectors were prepared by an external company (Viral Core Facility) by cloning the sequence of the MB22#2-GFP construct under the control of a cytomegalovirus (CMV) promoter. The lentivector (BL-0533) containing a GFP sequence under the same promoter was used as a control.

**Cell culture, transfection, and transduction**

The monkey COS7, human HEK293S, and mouse embryonic fibroblast (MEF) cells were grown in a high-glucose DMEM medium with L-glutamine (Biowest) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% antibiotic/antimycotic (Life Technologies) at 37°C in 5% CO<sub>2</sub>. Fibroblasts derived from patients with DM1 (cell lines GM04033 and GM03989 expressing CUG<sub>1,000</sub> and CUG<sub>2,000</sub> CUG repeats, respectively) and control fibroblasts derived from non-DM1 patient (cell line GM07492) were purchased from the Coriell Cell Repositories. Fibroblasts were grown in Eagle’s minimal essential medium (EMEM) (Biowest) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 1% antibiotic antymycotic (Life Technologies), and 1% non-essential amino acids solution (Sigma) in 5% CO<sub>2</sub> at 37°C. MEF 18K2KO cells were gifts from Maurice Swanson at the University of Florida. For transfection, cells were plated in 12-well plates and transfected at ∼80% confluence using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Single transfection with siRNA against the 3’ UTR fragment of the MBNL1 sequence or control siRNA (synthesized by Sigma-Aldrich) was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) at 50 nM concentration (sequence of siRNA duplexes are specified in Table S2). After 24 h, cells were transfected with plasmids. Co-transfection was conducted in a 1:1 ratio with MB22 construct and pEGFP-C1 or pEGFP-C1-MBNL1-41 vector; the same ratio was used in the
experiments with DT960 vector. The co-transfection with minigenes was made in a 1:10 ratio (minigene:construct). For all experiments, the total amount of DNA added to the cells was 1 µg/mL of cell culture medium, except for experiments with different plasmid concentrations, which are specified in figure legend (Figure 3B). The cells were harvested 48 h after transfection or 72 h after the experiment with siRNA treatment. The samples referred to as a mock are lipofectamine-treated cells. For transduction, cells were plated in 48-well plates. Lentiviral vectors with a concentration of 10^7 particles/mL at a multiplicity of infection (MOI) 1 for control and MOI 10 for MB22#2-GFP in an appropriate amount of growth medium were delivered into the cells. After 24 h, the medium was replaced. The cells were harvested after 12 days.

**Alternative splicing and real-time qPCR analysis**

The cells were harvested using TRIzol Reagent (Thermo Fisher Scientific), and total RNA was isolated using Total RNA Zol-Out D (A&A Biotechnology) according to the manufacturer’s protocol. cDNA was synthesized using TranScriba Kit (A&A Biotechnology) with Random Primers (A&A Biotechnology) according to the manufacturer’s protocol. PCR was performed using GoTaq DNA Polymerase (Promega), and primers are listed in Table S3. PCR products were separated in 1%–2% agarose gel with ethidium bromide. The images were captured using G:Box EF2 (Syngene) and analyzed using GeneTools (Syngene) (Figures S7A, S7B, S9A, S9B, S10, and S11). Percent sliced in (PSI) was calculated based on signals of two bands, corresponding to the PCR product containing or missing alternative exons, according to the following formula (isoform with included exon^100)/isoforms with included exon + excluded exon). Quantitative real-time RT-PCRs were performed in a QuantStudio 7 Flex System (Thermo Fisher Scientific) using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) according to the manufactures’ instructions. Targets were amplified with primers listed in Table S3 at 58°C–60°C annealing temperature. Ct values were normalized against GAPDH. Fold differences in expression level were calculated according to the 2−ΔΔCt method.

**Western blot**

Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid [EDTA], 0.5% NP-40, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were incubated on ice and vortexed followed by centrifugation at 15,000 × g at 4°C for 15 min. Concentration of protein in cell extracts were measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples were heated with standard sample buffer at 95°C for 5 min. Electrophoresis and wet transfer were performed with the use of the Mini-PROTEAN Tetra System (Bio-Rad). Protein extracts (25–40 µg) were separated on 10% SDS polyacrylamide gels in Laemmli buffer and transferred to nitrocellulose membrane (Sigma-Aldrich) (1 h, 100 V) in Laemmli buffer with 20% methanol. Membranes were blocked for 1 h in 5% Skim Milk Powder (Sigma-Aldrich) in TBST buffer (Tris-buffered saline [TBS], 0.1% Tween 20). Membranes were incubated with a primary antibody against FLAG (A8592, Sigma-Aldrich) 1:1,000, GAPDH (sc-47724, Santa Cruz) 1:10,000, or mCherry (5F8, ChromoTek) 1:1,000 in 5% Skim Milk Powder in TBST for 1 h at room temperature. Membranes were washed in TBST and incubated with secondary antibodies conjugated with horseradish peroxidase, anti-mouse (A9044, Sigma-Aldrich) 1:20,000, or anti-rat (ab6836, abcam) 1:8,000 in TBST for 1 h at room temperature. Membranes were again washed in TBST and detected using Immobilon Forte Western HRP substrate (Sigma-Aldrich). Images were captured using G:Box Chemi-XR5 (Syngene) (Figures S7B, S8, and S11) and quantified using Multi Gauge 3.0 software (Fujiﬁlm).

**Microscopic analysis**

The confocal microscopy was used to analyze sequestration of MBNL1auto-GFP to the CUG^gpp ribonuclear foci and to monitor homogeneity of the level of this protein in cells. COS7 cells were plated in µ-Slide 8 well (ibidi) after 48 h posttransfection with MB22#2-GFP, and (CUG^g1000) or (CUG^g0) medium was replaced with PBS with diluted Hoechst (Thermo Fisher Scientific), 1:2,000, and incubated for 5 min, protected from light. Images were captured with the Nikon A1Rsi confocal microscope with Nikon Apo ×40 WI λ 5 DIC N2 objective. GFP, Hoechst, and Cy3 were excited with 488 nm Argon-Ion and 405 and 561 nm diode lasers, respectively. For detection, dicroic mirrors of 405/488/561 nm with spectral filters of 525/50, 595/50, and 450/50 nm were used.

**Flow cytometry**

For flow cytometry experiments, cells were analyzed 48 h posttransfection with either GFP, MB22#2-GFP, or MBNL1-GFP plasmid. Culture medium was removed, and cells were washed with PBS, trypsinized, collected, and centrifuged for 5 min at 300 × g. Cell pellet was suspended in 400 µL PBS. The 100 µL cell suspension was diluted with 100 µL PBS and analyzed with guava easyCyteTM HT flow cytometer and guavaSoft software (Luminex). GFP fluorescence was excited by a 488 nm laser and detected at 525/30 nm. The threshold for GFP-positive cells was set based on the signal from mock-transfected cells. For each sample, 5,000 events were collected. The single event was referred to as single cell. The gating strategy is presented in Figure S3A.

**FISH**

RNA FISH and immunofluorescence (IF) cells were fixed in 2% PFA/PBS at room temperature for 10 min and washed three times in PBS. Pre-hybridization was performed in 30% formamide and 2× SSC for 10 min, followed by hybridization in buffer containing 30% formamide, 2× SSC, 0.02% BSA, 66 µg/mL yeast tRNA, 10% dextran sulfate, 2 mM vanadyl ribonucleoside complex, and 2 ng/µL DNA/LNA probe (CAG)30-CA. The probe was labeled at the 5’ end with Cy3 and modified at positions 2, 5, 8, 13, 16, and 19 with LNA. Posthybridization washing was done in 30% formamide and 2× SSC at 45°C for 30 min followed by 1× SSC at 37°C for the next 30 min. Slides were mounted in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) with DAPI.
Statistical analysis

Group data are expressed as the means ± standard deviation (SD). The statistical significance was determined by unpaired, two-tailed Student’s t test using Prism software v8 (GraphPad): *p < 0.05; **p < 0.01; ***p < 0.001; ns, non-significant. All analyses are based on at least three independent biological replicates (exceptions are indicated in the figure legends), and whole experiments were repeated at least twice to confirm obtained results.

DATA AVAILABILITY

This study did not generate/analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.10.023.

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AUTHOR CONTRIBUTIONS

Conception and design of the experiments, K.S.; performed the experiments, Z.R.; analysis of data, K.S. and Z.R.; writing and revising the manuscript and figures, K.S. and Z.R.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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