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Cooperation meets competition in microRNA-mediated DMPK transcript regulation

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ABSTRACT

The fundamental role of microRNAs (miRNAs) in the regulation of gene expression has been well-established, but many miRNA-driven regulatory mechanisms remain elusive. In the present study, we demonstrate that miRNAs regulate the expression of *DMPK*, the gene mutated in myotonic dystrophy type 1 (DM1), and we provide insight regarding the concerted effect of the miRNAs on the DMPK target. Specifically, we examined the binding of several miRNAs to the DMPK 3′ UTR using luciferase assays. We validated the interactions between the DMPK transcript and the conserved miR-206 and miR-148a. We suggest a possible cooperativity between these two miRNAs and discuss gene targeting by miRNA pairs that vary in distance between their binding sites and expression profiles. In the same luciferase reporter system, we showed miR-15b/16 binding to the non-conserved CUG repeat tract present in the DMPK transcript and that the CUG-repeat-binding miRNAs might also act cooperatively. Moreover, we detected miR-16 in cytoplasmic foci formed by exogenously expressed RNAs with expanded CUG repeats. Therefore, we propose that the expanded CUGs may serve as a target for concerted regulation by miRNAs and may also act as molecular sponges for natural miRNAs with CAG repeats in their seed regions, thereby affecting their physiological functions.

INTRODUCTION

The involvement of microRNAs (miRNAs) in the pathogenic mechanisms of many human diseases has become increasingly apparent. These endogenous ∼22-nucleotide-long non-coding RNAs are potent regulators of gene expression. They act post-transcriptionally and exert their regulatory effects mainly by binding to the 3′-untranslated region (3′ UTR) of target mRNAs, which results in mRNA deadenylation and decay, translational suppression or, rarely, mRNA cleavage (1,2). By targeting multiple transcripts and affecting the expression of numerous proteins, miRNAs engage in various biological pathways in cells (i.e., proliferation, differentiation, development, apoptosis, metabolism and neurodegeneration). The interaction between miRNAs and mRNAs is influenced by many factors; however, nucleotides 2–8 of the miRNA, termed the 'seed' sequence, are essential for target recognition and binding (1). The number and distribution of miRNA binding sites as well as plausible miRNA cooperation are particularly important. More than a decade ago, the insertion of multiple binding sites in reporter constructs used to validate miRNA–mRNA interactions has been suggested to ensure a higher efficiency of such constructs (3,4). It was later demonstrated that two sites in the same or different miRNAs could act synergistically and that the distance between neighboring miRNA binding sites affects the strength of the target down-regulation. Specifically, an optimal down-regulation was observed when the distance between the 3′ end of the first miRNA site and the 5′ end of the subsequent one was > 7 and < 40 nt (5), and when the 5′ ends of both miRNA seeds were separated by between 13 and 35 nt (6). More recently, the concept of miRNA synergy was reexamined and addressed in a more detailed way, showing that this mechanism of miRNA-mediated regulation can affect thousands of human genes. In a transcriptome-wide approach, it was demonstrated that miRNA sites spaced by a maximum of 26 nt may act cooperatively and that the human transcriptome is enriched for miRNA-binding sites located at a cooperativity-permitting distance (7).

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Moreover, a workflow for the identification and analysis of RNA triplexes composed of two cooperating miRNAs and a target mRNA has been proposed, and all of the predicted human target genes of synergistic miRNA regulation have been collected in the TriplexRNA database (8). The synergistic activity of co-expressed miRNAs as well as those that exhibit differential expression across tissues could be employed in therapeutic interventions using miRNA mimics and/or miRNA inhibitors. Interestingly, the natural antagonizing activity of two miRNAs with overlapping binding sites has been observed. More specifically, miR-184 was found to interfere with miR-205 in the suppression of the *SHIP2* gene. The binding of miR-184 to its seed sequence prevented the inhibitory effect of miR-205 on SHIP2 mRNA (9).

Myotonic dystrophy type 1 (DM1) is an incurable neuromuscular disorder that is caused by an expanded CTG*CAG repeat in the 3′ UTR of the *dystrophia myotonica-protein kinase* (*DMPK*) gene (10). The normal human *DMPK* gene harbors 5–37 copies of the trinucleotide motif, but a dynamic mutation may increase this number to over 5000 repeat copies. The expanded CUGs in DM1 result in the nuclear retention of mutant DMPK mRNA and reduced DMPK protein levels (11). Mutant transcripts sequester the muscleblind-like 1 (MBNL1) splicing factor, leading to the abnormal alternative splicing of a multitude of other transcripts and the expression of fetal forms of their protein products in DM1 adults (12,13). Spliceopathy is therefore thought to be the major factor underlying the pathogenesis of DM1. However, alternative mechanisms such as additional changes in gene expression, antisense transcripts, translation efficiency, misregulated alternative polyadenylation and miRNA deregulation may contribute to the pathogenesis of DM1 (14,15).

A few reports detailing a close connection between miRNAs and DM1 have been published (reviewed in (16)). The deregulation of specific miRNAs has been linked with muscular dystrophies and cardiomyopathies (17–19) and with myotonic dystrophy type 2 (DM2) (20). In DM1, alterations in the miRNA expression patterns have been observed in muscle-specific miRNAs (myomiRs). More specifically, in DM1 skeletal muscle, miR-1 and miR-335 are up-regulated whereas miRs 29b, 29c and 33 are down-regulated, compared with the control muscles (21). In addition, miR-1 is down-regulated in cardiac muscle (22), whereas miR-206 is up-regulated in the skeletal muscle of DM1 patients (23). The deregulation of DM1-associated miRNAs has also been linked to alterations in their putative target expression, indicating that miRNA misregulation in DM1 is functionally relevant and may contribute to the disease pathology (21,22). Importantly, the decreased expression of mature miR-1 and increased levels of its targets in the hearts of individuals with myotonic dystrophy are mediated by the functional depletion of MBNL1, a sequestered splicing factor, which affects the processing of pre-miR-1 (22). Recently, a study investigating a transgenic fly model of DM1 (i(CTG)480 *Drosophila* line carrying 480 CTG repeats) revealed that miRNA alterations were caused directly by CTG expansions (24). Specifically, the expression of 20 miRNAs was changed in DM1 flies compared with control flies; 19 were down-regulated and one was up-regulated. Taken together, the abovementioned reports indicate the pathological potential of miRNA dysregulation in DM1. However, regarding the possible treatment of DM1, of particular importance is a report showing that some miRNAs are predicted to preferentially bind and repress toxic transcripts with longer CUG repeats (25).

In this study, we focus on the miRNA-mediated regulation of the DMPK transcript, which provides a unique model for the investigation of miRNA binding in the context of potential miRNA cooperativity. Using a luciferase reporter system, we validated the regulation of the DMPK transcript by conserved miRNAs, miRs 206 and 148a, as well as miR-15b/16 binding to a non-conserved CUG tract. We demonstrated a possible cooperativity between the miR-206/148a pair and the potential for cooperative targeting of the CUG tract by CUG-repeat-binding miRNAs. In addition, we demonstrated the enrichment of miR16 in RNA foci composed of exogenously expressed CUG-repeat transcripts by RNA fluorescence *in situ* hybridization (RNA FISH), supporting the possibility of miRNA sequestration by the CUG repeats present in the DMPK 3′ UTR.

MATERIALS AND METHODS

Computational prediction of the miRNAs binding to the DMPK 3′ UTR

The pipeline for the computational prediction of miRNA binding to the DMPK transcript is presented in Figure 1. The following steps were taken: (i) finding the conserved sites of the miRNA families conserved among vertebrates predicted by any of three of the most commonly used miRNA prediction programs (TargetScan Release 6.2, DIANAmicroT v. 5.0 and miRanda August 2010 Release) (26–28); (ii) adding the poorly conserved miRNA–mRNA sites that were predicted by all of these programs; (iii) predicting additional miRNAs that would bind to the CUG repeats using an in-house method.

Identification of miRNAs that potentially bind to CUG repeats

Mature human and murine miRNA sequences were retrieved from miRBase (ver. 20) (29). In-house scripts written in the Python programming language were used to detect miRNAs with at least six complementary matches to CUG repeats in the same reading frame within the miRNA seed regions (positions 2–7 of the miRNA sequences).

Examination of the frequency distribution of binding sites for miRNA pairs

The sequences of human miRNA families with shared seed regions were obtained from TargetScan, Release 6.2 (30). The mature miRNA expression profiles (qPCR miRNA profiling) were obtained from miRNAMap 2.0 (31). Broadly expressed miRNAs were defined as miRNAs that were expressed in at least 10 of the 18 examined normal adult tissues at considerable levels (at least 2% of the total miRNA expression). miRNAs with at least 75% of the reads found in the tissue with the highest expression levels were classified as tissue-specific miRNAs. Next, miRNAs

were grouped into miRNA families (miRNAs with common seed regions), and only the miRNA families with all of the members nominated as broadly expressed or tissue-specific were considered to calculate the distances between putative miRNA binding sites (all of the distances were counted between the 5′ ends of the binding sites). Lists of miRNA families with broadly expressed and tissue-specific miRNAs can be found in the supplementary data (Supplementary Table S1). The distances between all of the distinctive pairs of miRNAs were used as reference controls. A Perl script provided by TargetScan was used to predict the miRNA target sites for the longest variants of the human 3′ UTR transcripts, and in-house scripts written in Python were used to process the data.

Cell culture

HEK 293T and HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 8% fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine and an antibiotic–antimycotic solution (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2. At 24 h before transfection, the cells were plated in 12-well or 6-well dishes in DMEM medium and harvested 24, 48, and 72 or 96 h post-transfection for the luciferase assay, real-time polymerase chain reaction (PCR), and western blot analyses, respectively.

Plasmid constructs and synthetic miRNA oligonucleotides

To generate reporter constructs bearing miRNA-binding sites, the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) was used. Specific oligonucleotides of different lengths with *Dra*I and *Xba*I restriction sites and containing single binding sites (b.s.) for the analyzed miRNAs (DMPK b.s. for miRs 1, 148a, 206 and combined 148a/206) as well as the oligonucleotides composed of 5 CUG, 20 CUG and 20 CAA repeats were synthesized (IBB Warsaw). The appropriate oligos were annealed and cloned into the pmirGLO vector, which was previously digested with *Dra*I (Fermentas) and *Xba*I (Fermentas) restriction enzymes downstream of the *luc2* gene. For all of the miRNAs, three types of constructs were prepared, namely wild-type (WT) constructs, constructs carrying mutations (MUT) predicted to disrupt binding and perfect match (PM) constructs, as previously described (32,33). The reporter constructs carrying 44, 53 and 72 CUG repeats were generated based on the reporter containing 20 CUGs via the SLIP (synthesis of long iterative polynucleotide) method (34,35). Briefly, the pmirGLO plasmid with the cloned sequence corresponding to 20 CUG repeats was independently digested with *Dra*I and *Sal*I restriction enzymes and subjected to one thermal cycle of 95°C for 5 min, 50°C for 10 min and 72°C for 3 min with 2.5 units of *Pfu* polymerase, reaction buffer, and 250 μM each dNTP. The product was directly transformed into *Escherichia coli* and selected on an LB plate containing ampicillin. The sequences of all of the constructs are presented in the supplementary data (Supplementary Table S2).

For miRNA overexpression, commercial plasmid constructs expressing miRNA precursors (pri-miR-1, pri-miR-206, pri-miR-214 (System Biosciences), pri-miR-148a and pri-miR-29a (Cell Biolabs)) were used. Moreover, the construct expressing the miR-15b/16 precursor was generated based on an empty plasmid (System Biosciences) by cloning the appropriate sequence at the *Eco*RI and *Not*I sites. The mir-15b/16–2 cluster that was flanked by a ∼250-nt sequence was amplified from the genomic DNA using PCR and the following primers: forward 5′-GACGCGGAATTC CGAAGCCATGGAATTGACTT and reverse 5′-GAAG CGGCCGCAAGAACAAAAACAAAGGAAAAGGA.

Cell transfection

HEK 293T and HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. For the luciferase assays, the cells were transfected in 12-well plates at ∼80% confluence. For each transfection experiment, 200 ng of the appropriate reporter construct and 400 ng of the appropriate miRNA-coding vector were used. For miRNA cooperativity studies, 200 ng of the appropriate reporters and a total of 400 ng of miRNA-coding plasmids (200 ng of either vector) were used. The cells were harvested 24 h after transfection and assayed for luciferase activity.

For the miRNA overexpression required for real-time PCR and western blot analyses, the HEK 293T cells were grown to 80% and 60% confluence, respectively, transfected in 12- and 6-well plates with 1 μg/ml pri-miRNA plasmid vectors, and harvested at 48 and 72/96 h, respectively.

For the fluorescence *in situ* hybridization (FISH) experiments, the HEK 293T cells were grown to 80% confluence on microscope slides in 12-well plates, transfected with 0.5 μg/ml plasmid vectors expressing DMPK exons 11–15 containing either 960 CUG or CAG repeats in exon 15 or with a control plasmid lacking the repeats (36). For miR-214 detection, the cells were additionally co-transfected with 30 nM of miR-214 mimic (synthetic oligonucleotides were purchased from IDT and prepared as described in (33)).

Luciferase reporter assay

After harvesting, the cells were lysed in a passive lysis buffer (Promega). The enzymatic activities of firefly and *Renilla* luciferases were measured using a Centro LB 960 luminometer (Berthold Technologies) and the substrates and procedures provided with a Dual-Luciferase Reporter Assay System (Promega). The values for firefly luciferase activity for every reporter construct were normalized to the corresponding values of *Renilla* luciferase activity to account for varying transfection efficiency. The relative expression values for all of the constructs were obtained by comparing their normalized luciferase activities with those of the control plasmid.

RNA isolation and real-time PCR

The total RNA from HEK 293T cells was isolated using TRI Reagent (MRC, Inc., BioShop) according to the manufacturer's instructions. cDNA was obtained from 500 ng

of total RNA using Superscript III (Life Technologies) and random hexamer primers (Promega). For subsequent quantitative real-time analyses, 50 ng of cDNA was used. Real-time PCR was performed with a LightCycler 480 II system (Roche) using TaqMan Gene Expression Assays and TaqMan Universal Master Mix II (Applied Biosystems). The results obtained for the assessment of DMPK mRNA levels were normalized to the levels of actin mRNA.

Northern blot analysis

High-resolution northern blot analysis was performed as previously described (37,38). Briefly, 25 μg of total RNA was extracted from HEK 293T cells and resolved in a 12% denaturing polyacrylamide gel in 0.5× TBE. The RNA was transferred to a GeneScreen Plus hybridization membrane (PerkinElmer) using semi-dry electroblotting (Sigma-Aldrich), immobilized by subsequent UV irradiation (120 mJ/cm^2) (UVP) and baking at 80°C for 30 min. The membranes were probed with specific DNA oligonucleotides (Supplementary Table S3) that were complementary to the annotated human miRNAs (miRBase). The probes were labeled with [γ ^{32}P] ATP (5000 Ci/mmol; Hartmann Analytics) using OptiKinase (USB). The hybridizations were performed at 37°C overnight in a PerfectHyb buffer (Sigma-Aldrich). The marker lanes contained a mixture of radiolabeled RNA oligonucleotides (17-, 19-, 21-, 23- and 25-nt in length). Hybridizations to U6 RNA provided loading controls. Radioactive signals were detected by phosphorimaging (Multi Gauge v3.0; Fujifilm).

Western blot analysis

After transfection with the appropriate miRNA-coding plasmids, HEK 293T cells were lysed with 1× PBS supplemented with protease inhibitor cocktail (Roche). A total of 25–30 μg of protein lysate was separated by 12% SDS-PAGE. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (Sigma). All of the immunodetection steps were performed on a SNAP id (Millipore) in PBS buffer containing 0.25% nonfat milk and 0.1% Tween 20, and the membranes were washed in PBS/Tween. For DMPK and GAPDH detection, the blots were probed with the primary mouse anti-DMPK (1:500, Millipore) and mouse anti-GAPDH (1:5000, Millipore) antibodies, respectively, and they were subsequently probed with biotinylated secondary antibodies (1:500 Sigma). The membranes were incubated with a streptavidin–AP conjugate (1:2000, Millipore), and the immunoreactive bands were visualized using the Sigma Fast BCIP/NBT kit (Sigma).

RNA FISH and microscopy

RNA FISH was performed in transiently transected HEK 293T cultured cells as previously described (39). Briefly, 48 h post-transfection, the cells were fixed in 4% PFA/PBS at 4°C and washed three times in PBS for 5 min each. Prehybridization was performed in 30% formamide and 2× SSC buffer for 10 min followed by hybridization in a 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 appropriate fluorescently labeled RNA or DNA/LNA probes. Specifically, the miR-16–5p probe labeled at the 5′-end with TYE665 (Cy5) and modified at positions 5, 10, 14 and 19 with locked nucleic acids (LNA) (Exiqon) was used in combination with the $(CAG)_7$ probe labeled at the 5′-end with FAM and modified with 2′-O-methyl at positions 1 and 2 (IDT). In parallel experiments, the miR-214 probe labeled at the 5′-end with FAM and modified with 2′-O-methyls (IDT) was used together with the $(CAG)_7$ probe labeled at the 5′-end with TYE665 (Cy5) and modified at positions 6, 12 and 17 with LNAs (Exiqon). The $(CTG)_7$ probe, which was used additionally as a control to detect CAG RNAs, was labeled at the 5′-end with TYE563 (Cy3) and modified as described for the $(CAG)_7$ probe. Post-hybridization washing was performed in 30% formamide and 2× SSC at 45°C for 30 min followed by 1× SSC at 37°C for 30 min. The slides were mounted in SlowFade Gold Antifade reagent with DAPI (Invitrogen) for further microscopy.

To determine the spatial interactions between the expanded repeat RNA foci and specific miRNAs, the same exposure was established for all of the images from a single experiment, and a z-stack that was sufficient to cover all of the foci was acquired for each cell with a slice thickness of 1 μm using a PL-Apo 63× oil objective in conjunction with a cooled AxioCam HRc camera; the images in one stack were overlaid and saved as TIFF files. Approximately 100 cells were randomly selected to characterize the mutant repeat RNA and miRNA interactions in transfected HEK293 cells. The FISH images were processed with LSM 510 software (Zeiss).

Statistical analysis

The experiments were repeated at least three times. The graphs were generated using GraphPad Prism 5 (GraphPad Software). The figures for the luciferase assays were generated after averaging the results from the repeated experiments for a particular construct. The values for the error bars (means with SEM) and the statistical significance were calculated using GraphPad Prism 5. The statistical significance of the luciferase reduction in the case of transfection with constructs carrying miRNA b.s. was assessed using a one-sample *t*-test after checking that the data followed a normal distribution, with a hypothetical value of 100% assigned to the cells that were transfected with an empty vector control. *P*-values < 0.05 (two-tailed) were considered significant.

RESULTS

In our previous study, we performed an in-depth computational analysis of the miRNA interactions using all of the mRNAs derived from genes that trigger hereditary neurological disorders, known as trinucleotide repeat expansion diseases (TREDs), and we showed that *DMPK*, the gene that is mutated in DM1, may be subject to miRNA regulation (40). In the present study, following our guidelines for the efficient prediction of miRNA–mRNA binding sites (miRNA b.s.), we selected both highly and poorly conserved sites for experimental validation (Figure 1).

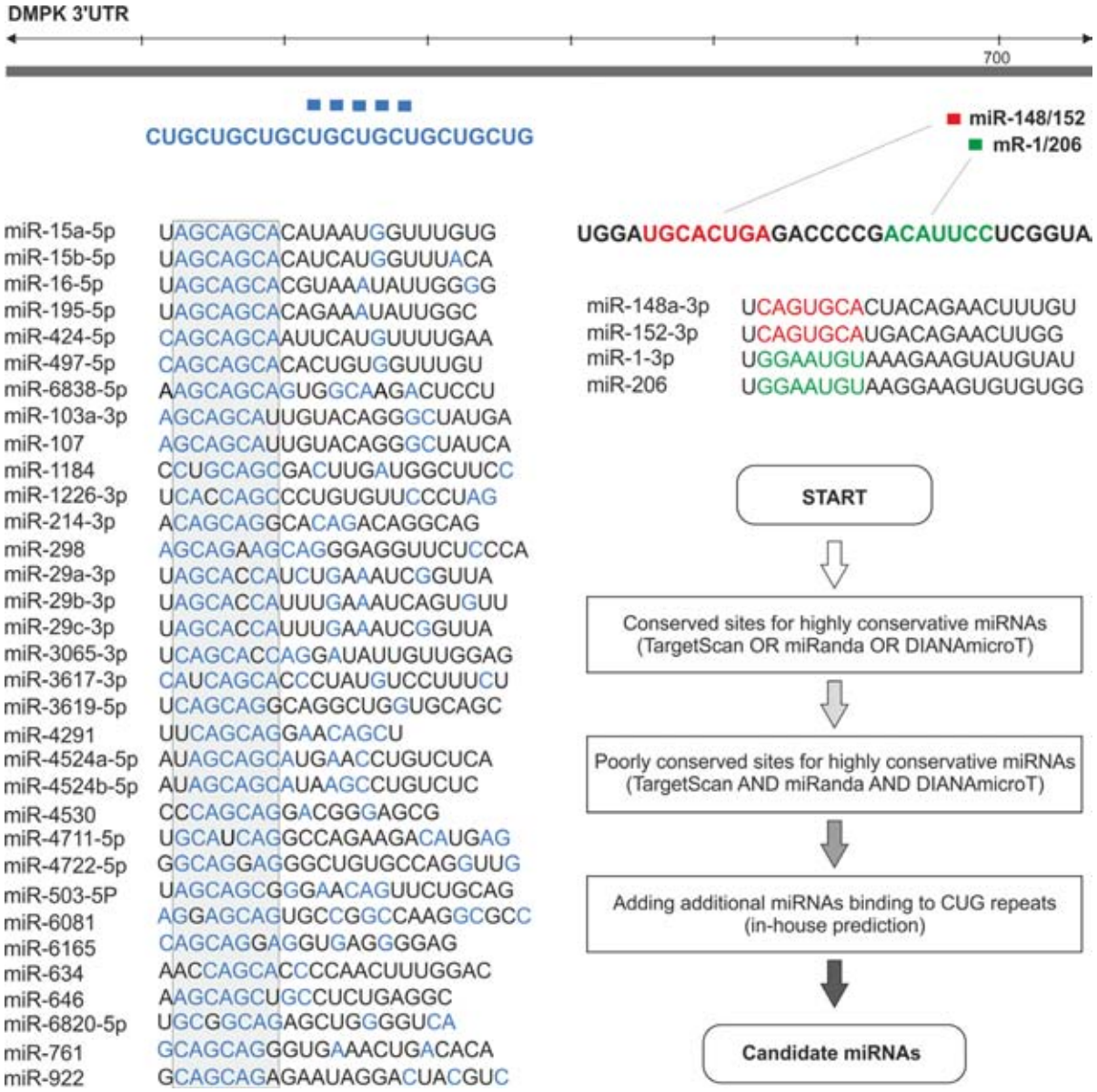

Figure 1. Analyzed miRNAs targeting the 3′ UTR of the DMPK transcript. A schematic presentation of the selected miRNA target site distribution in the DMPK 3′ UTR is shown. Additionally, a list of the miRNAs with CAG motifs in their seed regions that are complementary to the CUG repeats in the DMPK 3′ UTR and a pipeline for the computational prediction of the miRNAs that bind to the DMPK 3′ UTR are presented.

Regulation of the *DMPK* gene by the conserved miR-206 and miR-148a

The top candidate miRNAs (miR-148a/152 and miR-1/206) that are predicted regulators of the DMPK transcript (40) were ranked highly by the most commonly used algorithms to rank miRNA b.s., based on sequence conservation criteria. Both of the putative sites within the DMPK 3′ UTR were the only two highly conserved sites for miRNA families that are broadly conserved among vertebrates. The miRs 148a/152 and 1/206 exhibit disparate expression patterns in human tissues. Both miR-1 and miR-206 are myomiRs, which are abundantly expressed in smooth, skeletal, and/or cardiac muscles, whereas miR-148a and miR-152 are broadly expressed across various tissue types (31) (see Supplementary Table S1). The binding parameters for these candidate miRNAs meet the recommended bioinformatics criteria, and their experimental validation was of particular interest in the context of the pathogenesis and therapy of DM1 (22).

Experiments employing a set of reporter constructs and luciferase assays were performed to experimentally verify the predicted binding of miR-206 and miR-148a to their target sites within the 3′ UTR of the DMPK, as previously described (32,33). Briefly, the following constructs were tested in parallel: wild-type reporters (WT) bearing a single native b.s. for either miRNA, constructs with mutations (MUT) that disrupt the 5′ seed site (negative controls) and constructs showing perfect complementarity (PM) to miRNA b.s. (positive controls). Having performed a north-

ern blot analysis, which revealed that miR-206 was not expressed in HEK 293T cells and miR-148a was expressed at a low level (Figure 2A), we validated the predicted miRNA–mRNA interactions using a miRNA overexpression system. Specifically, HEK 293T cells were co-transfected with a reporter construct (carrying a potential b.s. for the studied miRNA) and the appropriate miRNA-coding plasmid (System Biosciences, Cell Biolabs). The transient transfection of the cells was followed by measuring the reporter activity.

We obtained considerable repression of luciferase expression following the transfection of both of the WT reporters, namely the WT construct carrying the b.s. for miR-206 (WT_206) and the WT construct carrying the b.s. for miR-148a (WT_148a) (Figure 2B). This result indicates that both miR-206 and miR-148a are functional and may down-regulate the DMPK transcript. The effect exerted by these two miRNAs was comparable, with miR-148a being slightly more effective. Specifically, the reduction of luciferase activity was reproducible and statistically significant for both WT constructs, with suppression to 81% and 71% for the WT construct carrying the b.s. for miR-206 and miR-148a, respectively. Both of the PM constructs repressed the luciferase activity to very low levels (13–16%), whereas the luciferase activity for both of the MUT constructs exhibited efficient de-repression (≥90%). To validate our experimental approach, we performed additional luciferase tests in HeLa cells and also HEK 293T cells but without the addition of the appropriate miRNA-coding vectors (Supplementary Text 1, Supplementary Figure S1A, S1B). The obtained results verified the reliability of the experimental system used. Thus, the presented study provides the first evidence of the direct binding of miRs 206 and 148a with the DMPK 3′ UTR and positively validates these miRNAs as negative regulators of the *DMPK* gene.

Next, we evaluated the expression of the DMPK protein and mRNA following the transfection of HEK 293T cells with plasmids encoding either miR-206 or miR-148a. Real-time PCR performed after transfection of the studied miRNAs did not reveal a considerable decrease in the DMPK mRNA (Figure 2C). Although miRNA binding frequently leads to the reduction of the cellular levels of targeted transcripts (41,42), our observation is consistent with the findings that no or minimal changes in the respective mRNA levels were observed or that these changes were only reported for certain targets (43). By contrast, western blot analyses performed with either miR-206 or miR-148a revealed a considerable decrease in the DMPK protein level, which was slightly more evident following the overexpression of miR-206 (Figure 2D). In muscle cells, which are the primary cell type affected in DM1, miR-206 is highly expressed. Therefore, to confirm the DMPK down-regulation observed in HEK 293T cells after miR-206 overexpression, we also addressed the regulation of DMPK expression in human muscle cells including DM1 myoblasts (Supplementary Text 2 and Figure S2). The observed increase of miR-206 expression, together with the decrease of DMPK protein levels, is in agreement with the hypothesis that miR-206 act as a negative regulator of DMPK expression.

Finally, we aimed to analyze the binding specificity of miR-1 and miR-206, which share the seed sequence and are predicted to bind to the same site in the DMPK transcript, but differ in four nucleotides at their 3′ ends (Figure 3A). Both miR-1 and miR-206 are widely studied and well-defined myomiRs; they enhance skeletal muscle differentiation, regulate numerous targets and are involved in a wide array of specific muscular pathways (reviewed in (44,45)). Moreover, these two miRNAs exhibit different expression patterns, depending on the muscle type, and differentially regulate transcripts that bear their b.s. (46). Although canonical miRNA-target specificity is triggered primarily by complementarity within the seed region, non-canonical interactions also depend on 3′ compensatory sites (1,47). To determine whether miR-1 and miR-206 differ in their regulatory potential, we performed a relevant luciferase experiment. We co-transfected HEK 293T cells with the reporter construct carrying the potential b.s. for miR-1 (WT_1) and a miR-1-encoding plasmid (System Biosciences) together with the adequate controls. In contrast to the repressed luciferase expression that followed the transfection of the WT_206 construct, after transfection of WT_1, we observed only a slight decrease in luciferase activity (suppression to 92%) (Figure 3B). There was no visible decrease in the protein level of DMPK and no statistically significant reduction of the mRNA level following miR-1 overexpression (Figure 3C, D, E). These results suggest that miR-1 is a weaker regulator of DMPK expression than miR-206; however, it cannot be ruled out that, under certain conditions, this miRNA may affect the expression of DMPK to variable degrees.

Potential cooperativity between miR-206 and miR-148a

Having validated the individual interactions of miR-206 and miR-148a with the DMPK 3′ UTR, we proceeded with the same type of experiments to determine the spacing requirements for cooperative miRNA target site interactions. The distance between the miR-206 and 148a sites in the intact DMPK 3′ UTR measured between the 5′ ends of both seeds is 15 nt, which is considered to be the optimal spacing for efficient target repression (5–7). Additionally, this miRNA pair was predicted *in silico* to have the potential to act cooperatively, as measured by the energy gain achieved through RNA triplex formation in comparison to two separate miRNA–mRNA duplexes using the TriplexRNA database (8). To study the possible co-regulation of DMPK expression by the miR-206/148a pair, new sets of reporter constructs were prepared that differed in the distance between the b.s. of miR-206 and miR-148a. Three reporters were constructed: (i) a wild-type reporter bearing the native sequence of the DMPK 3′ UTR encompassing the b.s. of both miRNAs (WT_148/206); (ii) a reporter containing the sequence between miRNA seeds that were artificially extended to 31 nt (Ext_148/206), a nt length that is considered to promote miRNA cooperativity (6) and (iii) a reporter in which the sequence between miRNA seeds was extended to 85 nt (Long_148/206), a nt length that is considered to possess very limited miRNA cooperativity (Figure 4A). In the case of the Ext_148/206 construct, additional nucleotides were introduced to maintain a native sequence context, which is required for a proper interaction with both miR-206 and miR-148a. In addition, an *Eco*RI restriction site was introduced, permitting the sub-

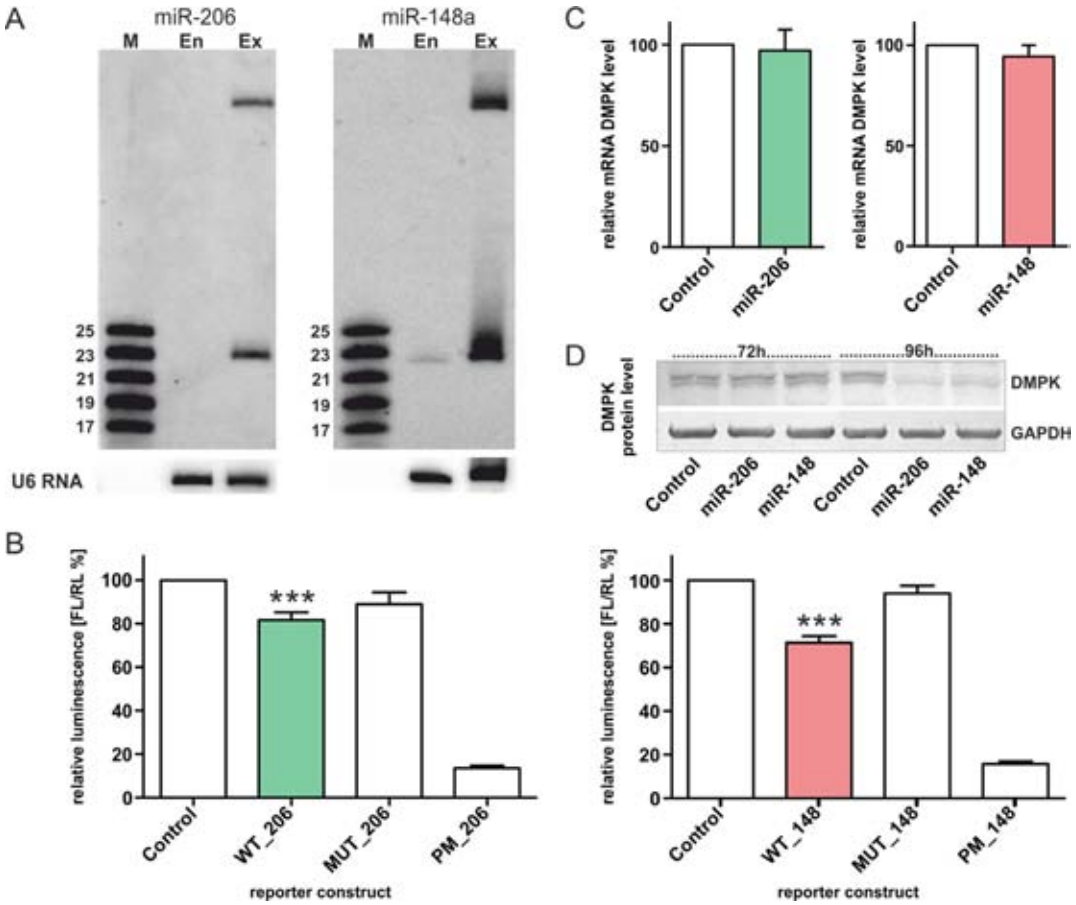

Figure 2. Regulation of the *DMPK* gene by miR-206 and miR-148a. (**A**) Northern blot detection of miRs 206 and 148a in untreated HEK 293T cells and cells that were transfected with miRNA-coding plasmids (System Biosciences, Cell Biolabs). M denotes the size marker: end-labeled 17, 19, 21, 23 and 25-nt oligoribonucleotides. En and Ex indicate the endogenous and vector-expressed miRNA levels, respectively. Hybridization to U6 RNA served as a loading control. (**B**) Relative repression of luciferase expression. Reporter constructs carrying a single b.s. for miR-206 and miR-148a were tested. For each luciferase experiment, the miRNA activity on four constructs was measured in parallel: an empty pmirGLO vector (Control), a wild-type potential b.s. for the appropriate miRNA (WT), a mutated b.s. (MUT), and a site with full complementarity (PM). The firefly luciferase activity was normalized against that of *Renilla* luciferase. The standard errors were calculated from nine independent experiments. The asterisks indicate statistical significance at $P < 0.001$. (**C**) Relative DMPK mRNA levels. Real-time PCR was performed after the HEK 293T cells were transfected with miR-206 and miR-148a. The bar graphs show the quantification of the DMPK mRNA levels normalized to the actin mRNA level, based on the data from five independent experiments. (**D**) Western blot analysis of the DMPK protein levels 72 and 96 h after the HEK 293T cells were transfected with miR-206 and miR-148a, as indicated. The GAPDH protein served as a loading control.

sequent extension of the spacing between these miRNAs b.s. In the case of the longest reporter construct, namely Long_148/206, an appropriate DNA fragment comprising an irrelevant sequence that further separated the sites for miR-206 and miR-148a was cloned into the *Eco*RI site. The secondary structures that can be formed by transcripts from every reporter construct, as predicted by the RNA metaserver available on the GeneSilico website, showed a high degree of secondary structure preservation in the miRNA b.s. (Figure 4B).

Luciferase assays were performed as described to validate the individual miRNA–mRNA interactions. The reporters bearing two miRNA sites with increasing separations, i.e., WT, Ext and Long, were tested in parallel following the transfection of HEK 293T cells and the simultaneous overexpression of both miRNAs (an equal amount of the plasmids encoding miRs 148a and 206 was transfected into the cells). Importantly, all of the analyzed constructs significantly suppressed luciferase expression, demonstrating their ability to bind miRNAs, but their efficiency varied (Figure 4C). Specifically, after the overexpression of both miRNAs, the WT construct carrying the native sequences of the miR-148a and miR-206 sites (WT_148/206) reduced the luciferase activity to 66% compared with the control, which is a better score compared with that observed for the down-regulation resulting from the activity of either individual miRNA (compare with Figure 2B and Supplementary Figure S1A). However, in the case of the Ext_148/206 construct with the miRNA site separation extended to 31 nt, the reduction of luciferase activity was much stronger (suppression to 42%). By contrast, in the case of the Long_148/206 construct with miRNA site separation extended to 85 nt, the reduction of luciferase activity was again at a level similar to that produced by the WT_148/206 construct (suppression to 65%). The obtained results indicate that miRNAs 148a and 206 may act cooperatively and that their optimal

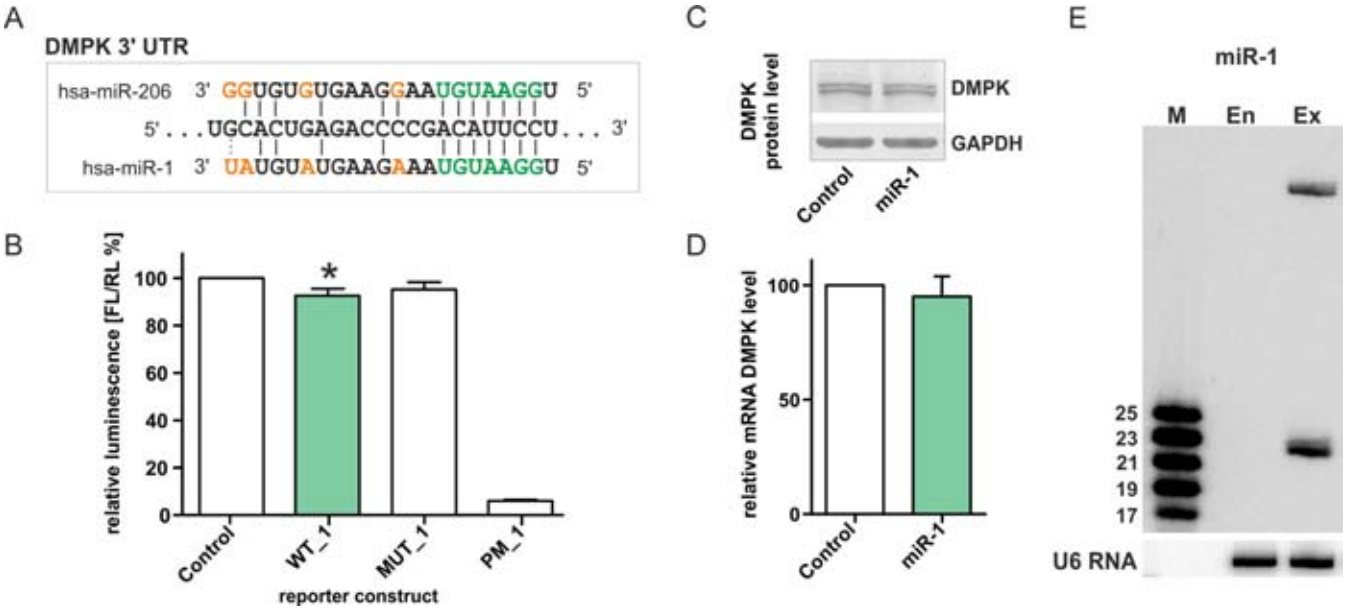

Figure 3. Regulation of the *DMPK* gene by miR-1. (**A**) Schematic presentation of the base pairing of miR-1 and miR-206 with their target DMPK sequences. The nucleotides in the seed regions of these miRNAs are marked in green, whereas disparate nucleotides are shown in orange. (**B**) Relative repression of luciferase expression. Reporter constructs carrying a single b.s. for miR-1 were tested; the miRNA activity on four constructs was measured in parallel (Control, WT, MUT and PM) as described in Figure 2. The standard errors were calculated from eight independent experiments. The asterisk indicates statistical significance ($P < 0.05$). (**C**) Western blot analysis of the DMPK protein levels after the HEK 293T cells were transfected with miR-1. The GAPDH protein served as a loading control. (**D**) Relative DMPK mRNA levels. Real-time PCR was performed after the HEK 293T cells were transfected with miR-1. The bar graph shows the quantification of the DMPK mRNA level normalized to the actin mRNA level, based on the data from four experiment repeats. (**E**) Northern blot detection of miR-1 in the untreated HEK 293T cells and cells that were transfected with the miR-1-coding plasmid (System Biosciences). M denotes the size marker: end-labeled 17, 19, 21, 23 and 25-nt oligoribonucleotides. En and Ex indicate the endogenous and vector-expressed miRNA levels, respectively. Hybridization to U6 RNA served as a loading control.

distance between b.s. is in agreement with that previously studied in other human miRNAs (5–8).

The cooperativity between miR-206 and miR-148a in the regulation of DMPK could be further confirmed by the observation that the expression of both the DMPK protein and mRNA was affected following the simultaneous overexpression of the cooperating miRNAs in HEK 293T cells (Figure 4D, E). Interestingly, the real-time PCR results revealed a minor decrease in the DMPK mRNA level after transfection of the miR-148a/206 pair, which was statistically significant and greater than the effect exerted by a single miR-148a or miR-206 (Figure 2C). It is worth noting that the effect of miRNA cooperativity was achieved when a half concentration of each miRNA was used (see Materials and Methods). The DMPK protein was considerably repressed when miR-206 and miR-148a were mildly but simultaneously overexpressed in HEK 293T cells, whereas the protein level was not decreased when a lower concentration of either of the single miRNAs was used (Supplementary Figure S3).

The RNA structure itself does not seem to be the main factor influencing the potential miRNA binding; however, spatial separation and a rather loose structure are preferable (48–50). To assess the impact of the secondary structure of the target site on the cooperative binding of the miRNAs, an impaired reporter construct was also tested, namely the impaired Ext_148/206 (hereafter referred to as impExt_148/206). The impairment of this reporter concerned its sequence; however, this change in sequence led to structural consequences. Specifically, nucleotides in the region of the miRNA b.s. were predicted to be more tightly paired, and a stem rather than a loop was formed at the miR-206 b.s., resulting in the lower efficiency of this construct compared with Ext_148/206. After the transfection of impExt_148/206, the luciferase expression decreased to 85% (Supplementary Figure S4), which was rather similar to the reduction achieved by the overexpression of only one miRNA, suggesting a loss of miRNA cooperativity. In the same experimental setup, the Ext_148/206 reporter could suppress luciferase up to 42% (Figure 4C).

Global analysis of the miRNA–mRNA binding sites

Although a few significant advances in the field of miRNA cooperativity have been made (5–8), neither its mechanism nor its biological relevance is yet fully understood. Therefore, after discovering the co-regulation of the DMPK transcript via the ubiquitously expressed miR-148a and tissue-specific miR-206, we investigated whether this type of miRNA cooperativity in the regulation of gene expression could be a common phenomenon that is dependent on the miRNA expression profiles, i.e., whether broadly expressed miRNAs and tissue-specific miRNAs could work in tandem.

Using the qPCR expression profiles of human miRNAs in different tissues (miRNAMap 2.0, (31)), we selected miRNA families in which every member of the family was ubiquitously expressed (94 miRNA families) as well as a set of tissue-specific miRNA families (25 miRNA fami-

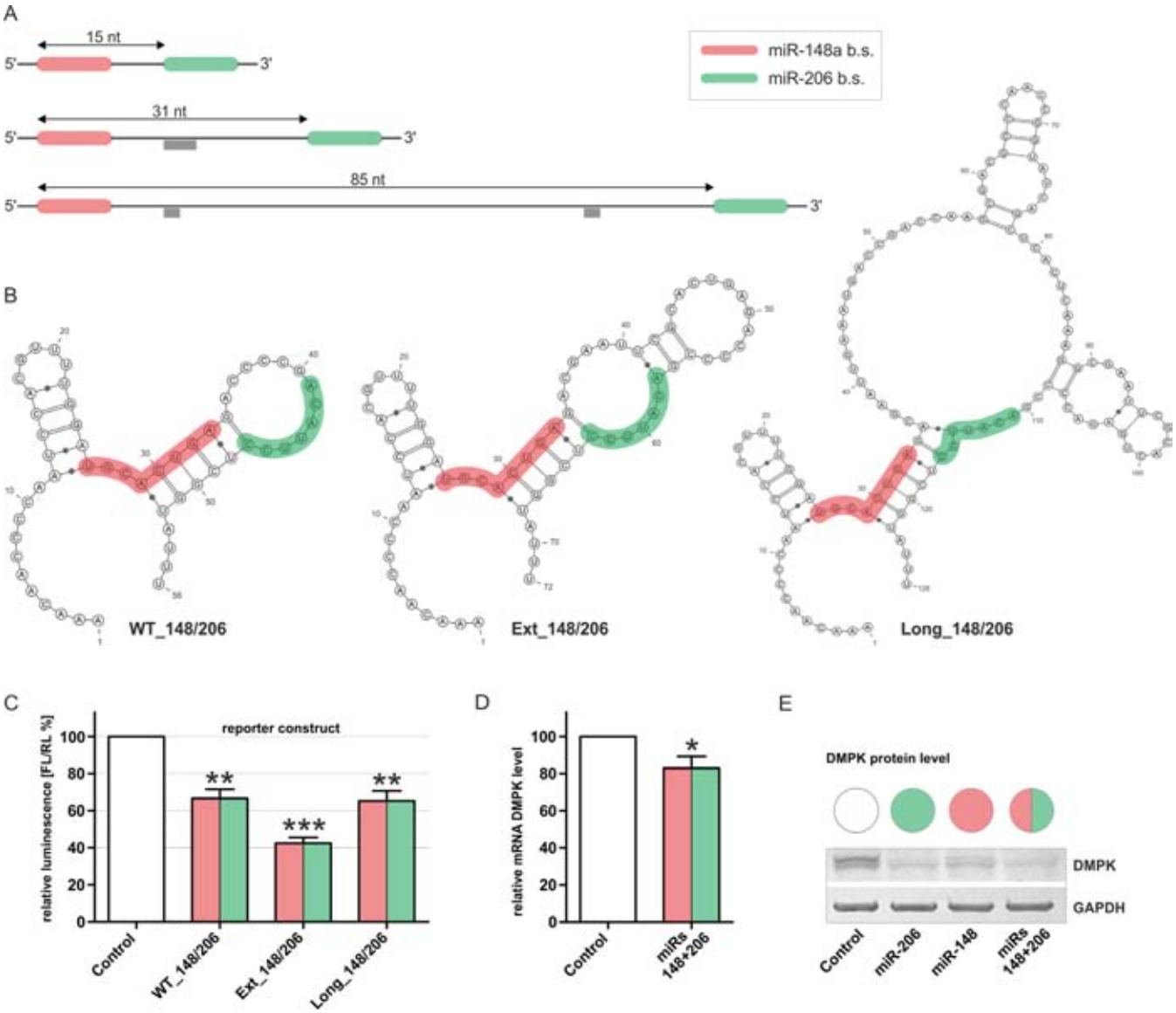

Figure 4. Potential cooperativity between miR-206 and miR-148a. (**A**) A schematic presentation of the reporter constructs that were used to study the role of the distance between the b.s. of miR-206 and miR-148a. Three types of reporters are shown with the 5′ ends of the miRNA b.s. separated by 15, 31 and 85 nt. The miR-148a and miR-206 seed regions are marked in light red and light green, respectively. (**B**) Graphical representation of the secondary structures formed by the reporter constructs of different lengths as predicted by RNAmetaserver. WT_148/206, Ext_148/206 and Long_148/206 correspond to constructs with miRNA b.s. spaced by 15, 31 and 85 nt, respectively. (**C**) Relative repression of luciferase expression by constructs that differ in the distance between the b.s. of miRs 148a and 206. The miRNA activities on the four constructs were assessed in parallel (Control, WT_148/206, Ext_148/206 and Long_148/206) after the simultaneous overexpression of both miRNAs. The standard errors were calculated from six independent experiments. The asterisks indicate statistical significance; a double asterisk denotes $P < 0.01$, and a triple asterisk denotes $P < 0.001$. (**D**) Relative DMPK mRNA levels. Real-time PCR was performed after the simultaneous overexpression of miR-148a and miR-206 in HEK 293T cells. The bar graph shows the quantification of the DMPK mRNA level normalized to the actin mRNA level, based on the data from five independent experiments. (**E**) Western blot analysis of the DMPK protein levels after the simultaneous overexpression of miR-148a and miR-206 in HEK 293T cells. A representative blot is shown. The GAPDH protein served as a loading control.

lies) (see Materials and Methods and Supplementary Table S1). Next, for all of the human transcripts in the TargetScan database Release 6.2 (see Materials and Methods), we calculated the distances between the putative b.s. for three types of miRNA pairs: (i) pairs with one broadly expressed miRNA and one tissue-specific miRNA; (ii) pairs with two different tissue-specific miRNAs and (iii) pairs with two different broadly expressed miRNAs. Our analysis revealed different preferences for the distances between the three types of miRNA pairs examined (Figure 5). For the pairs of miRNAs with one broadly expressed miRNA and one tissue-specific miRNA, we observed an enrichment for overlapping sites (spacing of less than 7-nt, which causes the sequence of the 'seed' region to be shared by two miRNAs), but no enrichment of pairs with 7–28-nt spacing, the distance that enabled cooperativity of miR-148a and miR-206 in the DMPK transcript. This phenomenon suggests that there may be a tendency for tissue-specific miRNAs

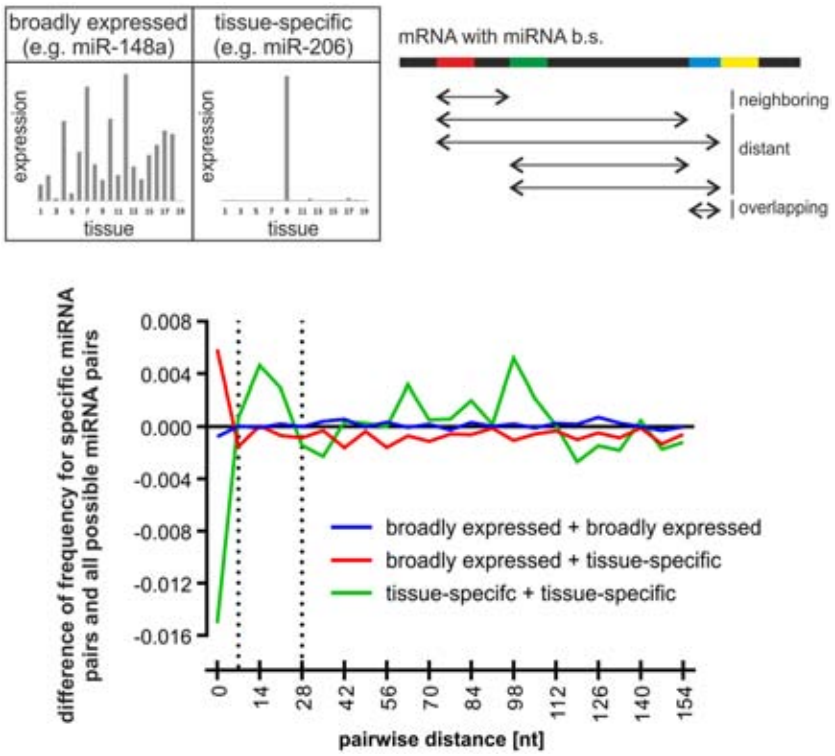

Figure 5. Distribution of the frequencies of the pairwise distance between the predicted binding sites of miRNA pairs. The graph shows the distribution of the b.s. distance frequencies between pairs with two broadly expressed miRNAs (blue line), two tissue-specific miRNAs (green line) or one broadly expressed miRNA and one tissue-specific miRNA (red line) in relation to the distribution of the miRNA b.s. distances for all of the miRNA pairs. The dotted lines indicate the 7- and 28-nt pairwise distances. In the upper panel, the schematic demonstrates how the different miRNA pairs were classified.

to regulate gene expression by competing with broadly expressed miRNAs for binding sites rather than via cooperative regulation by binding to the same transcripts in close vicinity. This competition for binding sites could be an additional mechanism for providing and maintaining tissue specificity. By contrast, we observed an enrichment of pairs of miRNAs with two tissue-specific miRNAs with 7–28-nt spacing. This result could be explained by the observation that tissue-specific miRNAs, which are often expressed in similar types of tissues (brain-, muscle-, liver-, testes- and placenta-specific miRNA families), orchestrate the same developmental/physiological processes and miRNA cooperativity increases the effectiveness of miRNA regulatory mechanisms ((7,51) and references therein).

Interaction of miR-15b/16 with CUG repeats in the DMPK transcript

In our prediction of miRNA-mediated regulation of the *DMPK* gene, we also sought to explore the possibility of miRNA binding to CUG repeats, which are the underlying cause of DM1 when they are extensively expanded. Previous studies have postulated the existence of several miRNAs that can potentially bind to the CUG repeats present in the DMPK 3′ UTR (25). If these interactions occur, the CUG repeats could act as multiple overlapping b.s. for several miRNAs and therefore have unique miRNA cooperative potential. We searched *in silico* for novel human miRNAs that can interact with repeated CUG sequences. We found 33 miRNAs with at least six complementary matches to CUG repeats in their seed regions (Figure 1 and Supplementary Table S4). All of these miRNAs were predicted to have additional pairing sites to a CUG sequence. It is worth noting that the members of one miRNA family (miR-15ab/-16/-195/-424/-497/-6838) bear a common seed region that is fully complementary to the CUG repeats. The observations that miRNAs from the miR-15/107 group that share a 5′ AGCAGC sequence (52) play key roles in gene regulation and have overlapping targets (53,54) are also of relevance to this issue. These miRNAs are expressed at medium-to-high levels across many tissues, and members with the 7-nt common sequence AGCAGCA exhibit the highest expression levels (55).

In the last set of experiments, we examined whether the miRNAs that were identified *in silico* as bearing CAG repeats in their seed regions bind to the CUG expanded repeats in the DMPK transcript. We used the same experimental approach with a luciferase reporter system. The candidate miRNAs were chosen based on the number of matches with the CUG repeats in their seeds (Supplementary Table S4). Specifically, we selected miR-15b and miR-16 (seven matches) and miR-214 and miR-29a (six matches). First, we co-transfected HEK 293T cells with reporter constructs carrying either 20 CUG repeats (referred to as WT_CUG because there are 20 CUGs in the human *DMPK* gene sequence deposited in GenBank (NM_001081560.2)) or 20 CAA repeats (referred to as MUT_CUG) and an appropriate miRNA-expressing vec-

tor. In the luciferase assays, we obtained considerable and statistically significant repression of the luciferase expression only after transfection of the reporters and miR-15b/16 (luciferase repression equal to 62%). In the case of the other tested miRNAs, the luciferase activity was not repressed (Figure 6A).

Next, we aimed to determine whether the length of the CUG sequence correlated with the level of transcript down-regulation. Therefore, we prepared and tested reporter constructs that differed with respect to the number of CUG repeats: the reporters carried 5, 20 (WT), 44, 53 and 72 CUG triplets, the last two mimicking the expansion of CUGs that is observed in DM1 patients. Similarly, in the luciferase assays, we obtained significant repression of the luciferase expression after the transfection of miR-15b/16 with most of the reporters, with the exception of the reporter with 5 CUGs (Figure 6B). Moreover, a trend toward stronger repression was observed when constructs with a longer repeat length were used; the luciferase repression was equal to 64% and 44% of the expression levels for the constructs with 20 and 72 CUGs, respectively. By contrast, the luciferase activity was not noticeably reduced when other miRNAs were co-transfected along with the tested reporters, with the exception of miR-214. The latter considerably repressed luciferase activity, but only in the presence of the longest reporter (with 72 CUGs) (repression to 73%). This finding suggests that 7-mer CAG miRNAs may preferentially bind to and suppress the expression of transcripts containing CUG repeats. Overall, these results indicate that certain miRNAs can target DMPK mRNA with expanded triplets, and the degree of miRNA-mediated repression depends on the number of repeats. The interaction between the DMPK 3′ UTR and miR-15b/16 with a CUG binding motif in their seeds containing seven matching nucleotides (AGCAGCA), is a notable example. Interestingly, this interaction is even stronger in terms of the DMPK repression achieved by appropriate reporter constructs carrying 20 or more CUG repeats than the interaction between the analyzed conserved miRNAs (non-CUG-repeat-binding miRNAs), namely miRs 148a and 206, when expressed individually (compare with Figure 2B and Supplementary Figure S1A).

Some of the CUG-repeat-binding miRNAs are endogenously expressed in cells at high levels (55) (Figure 6C). In our experimental luciferase-based system, we overexpressed miRNAs from plasmids and ensured nearly equal concentrations of all of the tested miRNAs. However, the luciferase tests used to study miRNA binding to CUG repeats were also positive in the absence of miRNA overexpression (Figure 6D).

Next, we attempted to evaluate the expression of the DMPK protein and mRNA following the transfection of HEK 293T cells with plasmids encoding miRs 15b/16, 214 and 29a, as well as the positive control transfection with siRNAs specific to the DMPK sequence (56). The western blot and real-time PCR analyses did not show a considerable decrease in the DMPK protein and mRNA levels after transfection with the miRNAs (Figure 6E, F). Sequencing of the endogenous DMPK 3′ UTR fragment derived from the HEK 293T cells used here revealed that these cells harbored only 5 CTG repeats in the 3′ UTR of the *DMPK* gene, rather than the 20 CTGs anticipated based on the reference sequence deposited in GenBank. Therefore, this observation is in agreement with the results of the luciferase assay showing that the CUG-repeat-binding miRNAs cannot affect the control reporter bearing the 5 CUGs (see Figure 6B). To assess whether the presence of abnormally expanded CUG tracts may affect DMPK expression, we performed western blot analyses using fibroblast cells derived from DM1 patients (Supplementary Text 3 and Figure S6). We did not detect a significant change in the protein level in untreated cells (Supplementary Figure S6A), but the DMPK protein level was reduced after overexpression of miR-16 in fibroblasts with a CUG expansion (Supplementary Figure S6B), which may further confirm the possibility that miRNAs with CAG repeats in their seed regions are able to bind to the CUG repeats in the DMPK transcript.

Sequestration of miRNAs with a CAG sequence in their seeds by the expanded CUG repeats

Having examined the binding of miR-15b/16 and miR-214 to the reporter constructs bearing CUG triplet repeats, we studied the plausible sequestration of these miRNAs to the expanded CUG repeats exogenously expressed in human cells. We hypothesized that the presence of elongated CUG repeats would promote the binding of these miRNAs to the mutant DMPK transcript and that the sequestration of the CUG-repeat-binding miRNAs by abnormally lengthened mutant mRNAs could impair the miRNA-mediated silencing machinery. To determine whether miRs 16 and 214, which were previously tested in a reporter system, may be arrested by the CUG repeats present in the 3′ UTR of the truncated DMPK transcripts, we used fluorescence *in situ* hybridization (FISH) in HEK 293T cells as a model system in this study. We transfected HEK 293T cells with plasmid vectors expressing DMPK exons 11–15 containing either 960 CUG or 960 CAG repeats (36), or with the control plasmid lacking the repeats. The cells were also co-transfected with the miR-214 mimic; co-transfection with miR-16 was not necessary because it is expressed in HEK 293T cells at a high level (Figure 6C). This experimental approach permitted the simultaneous detection of both the selected miRNAs and the foci of either CUG- or CAG-triplet repeat mRNAs formed in the transfected cells. Using appropriate complementary probes that were fluorescently labeled with TYE665 (Cy5) or FAM at their 5′-ends (for details, see Materials and Methods), in the FISH experiment we detected that overexpression of 960 CUG repeat transcripts caused the formation of miR-16-enriched CUG RNA foci, in contrast to the uniform distribution of miR-16 in non-transfected cells (Figure 7A and Supplementary Figure S5). Interestingly, miR-16-containing CAG RNA foci were almost completely absent in cells overexpressing 960 CAG repeat transcripts (Figure 7B). This strongly suggests that miR-16 sequestration was specific for long CUG repeats. The vast majority of the miR-16-enriched RNA foci were found in the cytoplasm, where 68% were co-localized with the CUG RNA foci (Figure 7D). The relevant co-localization with CUG RNA inclusions was not observed for miR-214, the miRNA bearing six matches with CUG repeats in its seed (Figure 7C), or for the appropriate con-

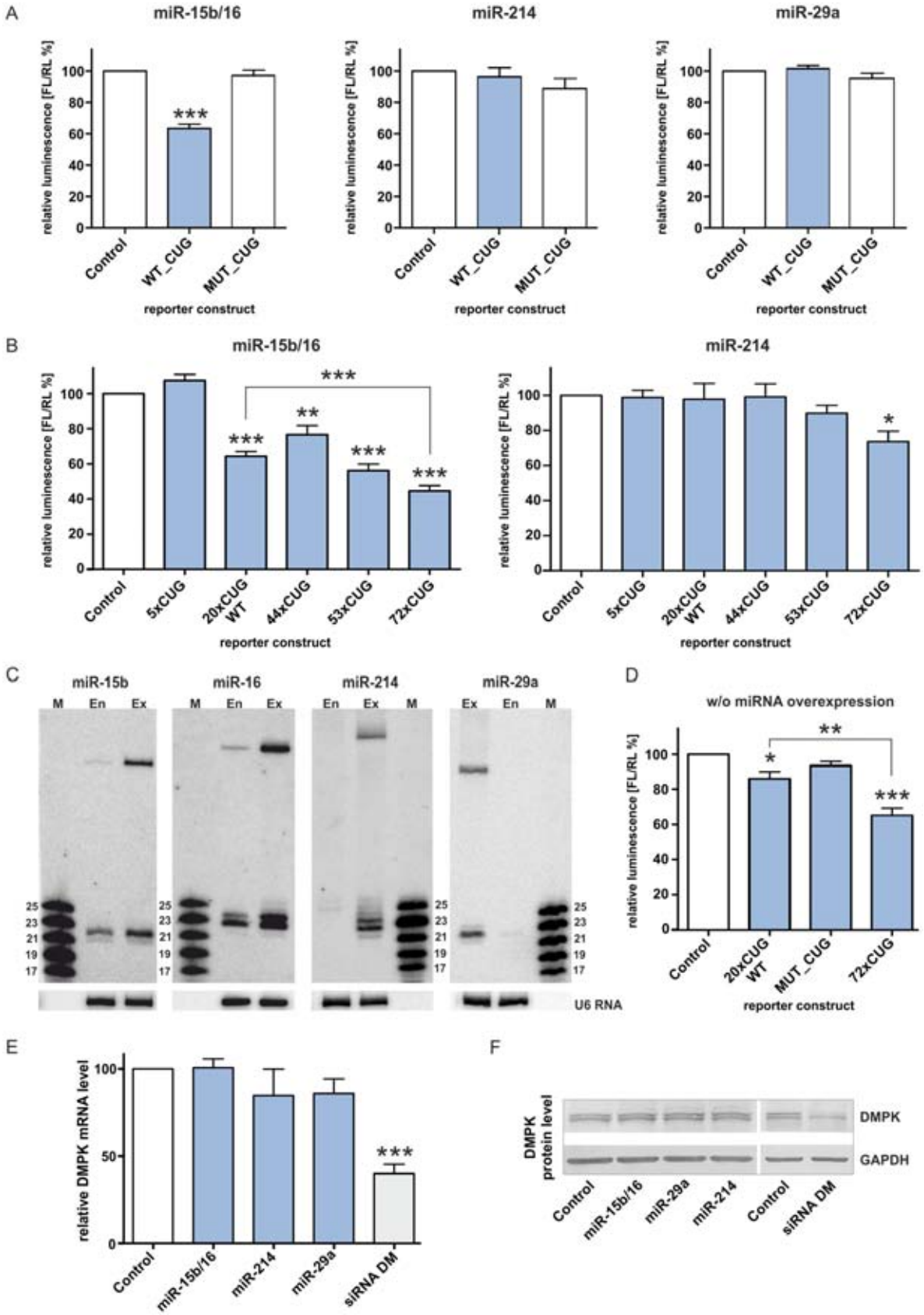

Figure 6. Interaction between miRNAs with CAG sequences in their seed regions and the CUG repeats in the DMPK transcript. (**A**) Relative repression of luciferase expression after transfection of reporter constructs carrying 20 CUG or 20 CAA repeats (denoted as WT_CUG and MUT_CUG) and either

trols. Therefore, the miR-16 co-localization with CUG foci detected by RNA FISH confirmed our results from the luciferase assays with the use of the same HEK 293T cells.

Expanded CUG repeat tracts form both nuclear and cytoplasmic aggregates (57,58); however, the pathogenicity associated with DM1 has been linked to the fact that mutant DMPK transcripts are retained in the cell nucleus (11,59–60). In our experimental system, in rapidly proliferating HEK 293T cells, we observed both nuclear and cytoplasmic CUG foci, but the co-localization of such foci with miR-16 occurred to a greater extent in the cytoplasm (most likely due to a cytoplasmic abundance of miR-16). Nevertheless, because miRNAs function in both the cytoplasm and cell nucleus (reviewed in (61)), we believe that our findings are relevant and further support the possibility of miRNA sequestration by the abnormally expanded CUG repeats in the mutant DMPK transcripts. More evidence for miRNA sequestration was indirectly provided by luciferase assays that were performed after the co-expression of appropriate reporter constructs and CUG-expanded repeats; an experiment with a reporter carrying a target sequence for miR-16 revealed that the presence of CUG repeats diminishes the miR-16 effect (Supplementary Text 4 and Figure S7).

Having experimentally demonstrated the binding of the miRNAs to CUG tracts using luciferase assays and having observed miR-16-enriched CUG RNA foci by RNA FISH, we sought to examine whether the enhanced interactions between the miRNAs and the aberrantly extended CUG repeats in the DMPK 3′ UTR could explain the changes in the transcriptome of DM1 mice reported by Thornton and colleagues (62). We computationally analyzed the miRNA regulation of genes with significantly altered expression in CUG transgenic mice (HSA^{LR}) in comparison with the parental line. First, analogous to the human miRNAs, we predicted the presence of murine miRNAs with CAG repeats in their seed sequences (Supplementary Table S5). Next, we compared the prevalence of the predicted conserved b.s. for those miRNAs in the 3′ UTRs of transcripts that are altered in CUG transgenic mice, and in the 3′ UTRs of all of the murine transcripts predicted to have at least one b.s. for any miRNAs using TargetScan Mouse Release 6.2. We observed an overall enrichment for transcripts bearing conserved miRNA b.s. in the CUG transgenic mice (78% of transcripts with at least one conserved b.s. versus 64% for all murine transcripts; $P = 0.0003$; Yates' continuity corrected chi-squared test). Moreover, we discovered a statistically significant enrichment for the former group, with 22% of transcripts with at least one b.s. for CUG-repeat-binding miRNAs versus 13% for all transcripts ($P = 0.0018$; Yates' continuity corrected chi-squared test) (Figure 8A and Supplementary Table S6). This trend was even stronger for transcripts that were uniquely deregulated in the CUG transgenic mice, but not in the *Mbnl1* or *Clcn1* knockout mice, which are alternative DM1 mouse models. Additionally, the transcripts that were uniquely deregulated in the $Mbnl1^{-/-}$ mice, but not in the HSA^{LR} mice, did not include any binding sites for the CUG-binding miRNAs. This observation supports the hypothesis that the miRNA deregulation observed in animal models of DM1 and DM1 patients may have a significant impact on changes in the transcriptome and may contribute to the pathogenesis of DM1.

DISCUSSION

The importance and role of miRNAs in human physiology and disease have been increasingly appreciated; however, many miRNA-driven regulatory mechanisms remain unclear. This study reports that miRNAs regulate the expression of the *DMPK* gene and examines how miRNAs function on a specific target sequence and on a repeated CUG sequence present in the DMPK transcript.

miR-206 and miR-148a regulate the DMPK transcript and may functionally cooperate

We experimentally assessed the validity of three predicted interactions with miR-1, miR-206 and miR-148a. Using luciferase reporter assays and sets of reporter constructs, we provide the first evidence of DMPK down-regulation by miR-206 and miR-148a. No changes were detected in the expression levels of the endogenous DMPK mRNA, but there was a significant decrease in the DMPK protein levels for both miR-148a and miR-206, which was even slightly higher for miR-206. Interestingly, in our experimental setup, we could not detect any regulation of the DMPK transcript by miR-1, which targets the same DMPK sequence and has the same seed as miR-206, but differs somewhat at the 3′ end. miR-206 has two additional Watson-Crick base pairs to the DMPK b.s., which are absent in the case of miR-1 binding. The variety in the 3′-end between miRs 1 and 206 seems to be important for their inhibitory activities against their targets. The impact of the

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the miR-15b/16-, miR-214- or miR-29a-expressing vectors, as indicated. The standard errors were calculated from twelve, eight and five independent experiments that were performed after overexpression of miRs 15b/16, 214 and 29a, respectively. The asterisks indicate statistical significance ($P < 0.001$). (**B**) Relative repression of luciferase expression by the reporters differing in the number of CUG repeats. Constructs with 5, 20 (WT), 44, 53 and 72 CUGs were tested in parallel. The luminescence was measured after the overexpression of miRs 15b/16 and 214, as indicated. The standard errors were calculated from ten and four independent experiments, respectively. The asterisks indicate statistical significance: a single asterisk denotes $P < 0.05$, a double asterisk denotes $P < 0.01$ and a triple asterisk denotes $P < 0.001$. (**C**) Northern blot detection of miRs 15b, 16 and 214 and 29a in untreated HEK 293T cells and cells transfected with the miRNA-encoding plasmids (System Biosciences). M denotes the size marker: end-labeled 17, 19, 21, 23 and 25-nt oligoribonucleotides. En and Ex indicate the endogenous and vector-expressed miRNA levels, respectively. The hybridization to U6 RNA serves as a loading control. (**D**) The relative repression of luciferase expression in HEK 293T cells measured after testing the reporter constructs carrying 20 CUG or 20 CAA repeats (denoted as WT_CUG and MUT_CUG) and 72 CUGs without miRNA overexpression. The standard errors were calculated from seven independent experiments. The asterisks indicate statistical significance: a single asterisk denotes $P < 0.05$, a double asterisk denotes $P < 0.01$ and a triple asterisk denotes $P < 0.001$. (**E**) Relative DMPK mRNA levels. Real-time PCR was performed after the HEK 293T cells were transfected with miRs 15b/16, 214, 29 and the appropriate siRNAs as a positive control (ssiDM10 (56)). The bar graphs show the quantification of the DMPK mRNA levels normalized to the actin mRNA level, based on the data from five independent experiments. (**F**) Western blot analysis of the DMPK protein levels after transfection of HEK 293T cells with miRs 15b/16, 214 and 29a, as indicated. The GAPDH protein served as a loading control.

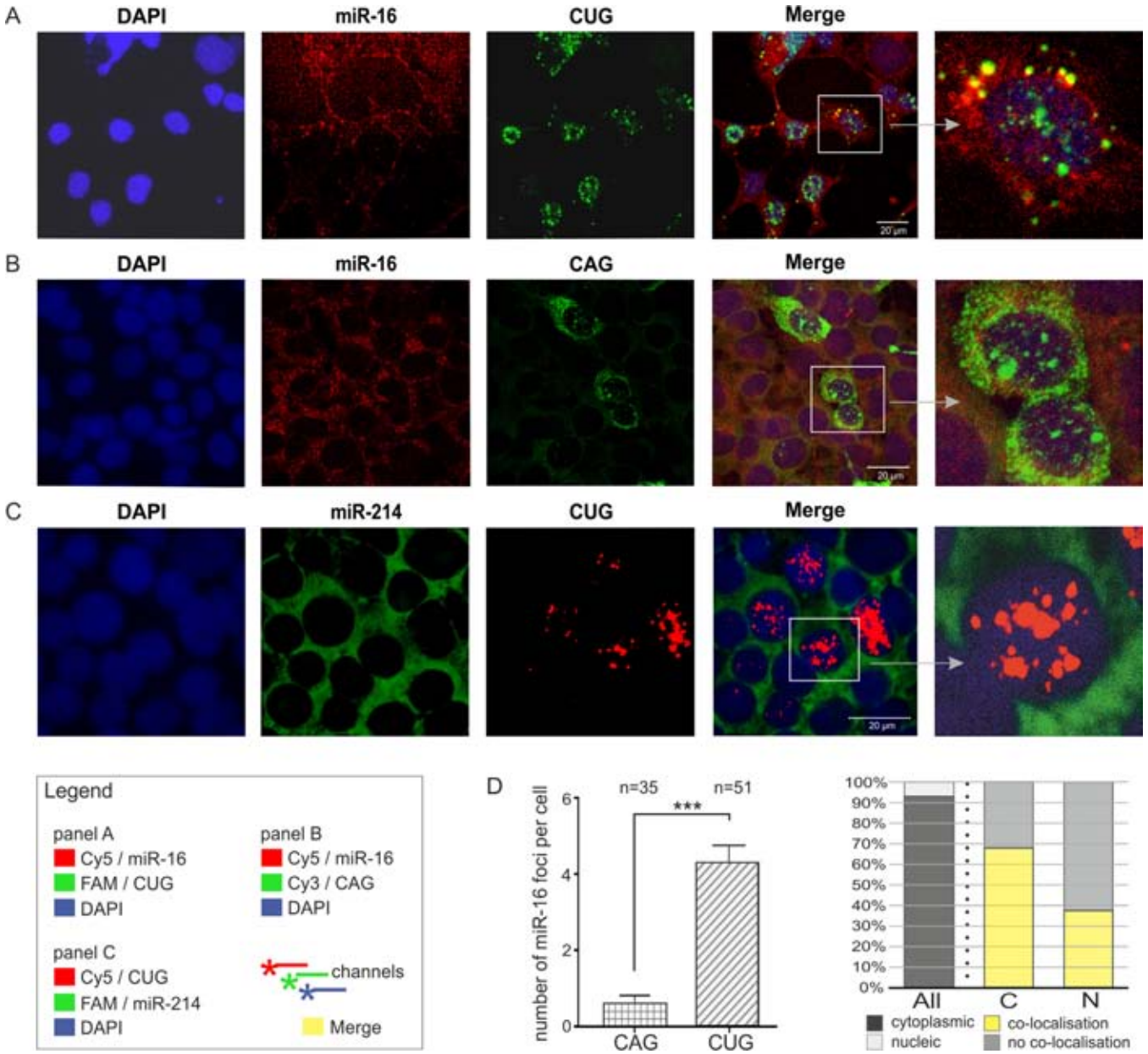

Figure 7. Visualization of miRNAs and cytoplasmic foci of CUG-triplet repeat mRNAs. (**A**) Representative RNA-FISH confocal images of cultured HEK 293T cells with endogenous miR-16 expression that were transfected with plasmid vectors expressing DMPK exons 11–15 containing 960 CUG repeats. (**B**) Representative RNA-FISH confocal images of cultured HEK 293T cells with endogenous miR-16 expression that were transfected with plasmid vectors expressing DMPK exons 11–15 containing 960 CAG repeats. (**C**) Representative RNA-FISH confocal images of cultured HEK 293T cells that were transfected with plasmid vectors expressing DMPK exons 11–15 containing 960 CUG repeats and the miR-214 mimic. The appropriate miRNAs and CUG repeat-containing RNAs were detected by differentially labeled fluorescent probes as depicted in the figure legend. The nuclei were stained with DAPI (blue). Scale bar: 20 μm. (**D**) Graphs showing the statistics for the estimation of the foci found in the cytoplasm (C) and nucleus (N), as well as information about the co-localization of the miRNA and CUG foci. The number of miR-16 foci in cells expressing 960 CUG and 960 CAG repeats were compared using the Mann Whitney test; ***$-P$-value < 0.001.

3′-end variability of the miRNAs on target recognition and binding strength has been previously reported. For instance, the functionality of the isomiR-214 variants, as opposed to the other tested miRNA isoforms, has been proposed to be due to compensatory base pairing at the 3′ end of the miR-214 isomiRs (33).

The phenomenon of miRNA cooperativity and/or synergy has not been fully elucidated. It was demonstrated that two sites of the same or different miRNAs could act synergistically; the optimal distance between two miRNA sites was determined to be approximately 7–40 nt between neighboring miRNA b.s. (5–7). Potential cooperativity between more distant miRNA sites has also been addressed (25,63–64); however, the optimal spacing requirements for neighboring miRNAs have not been precisely defined. In the present study, the distance between the miR-206 and

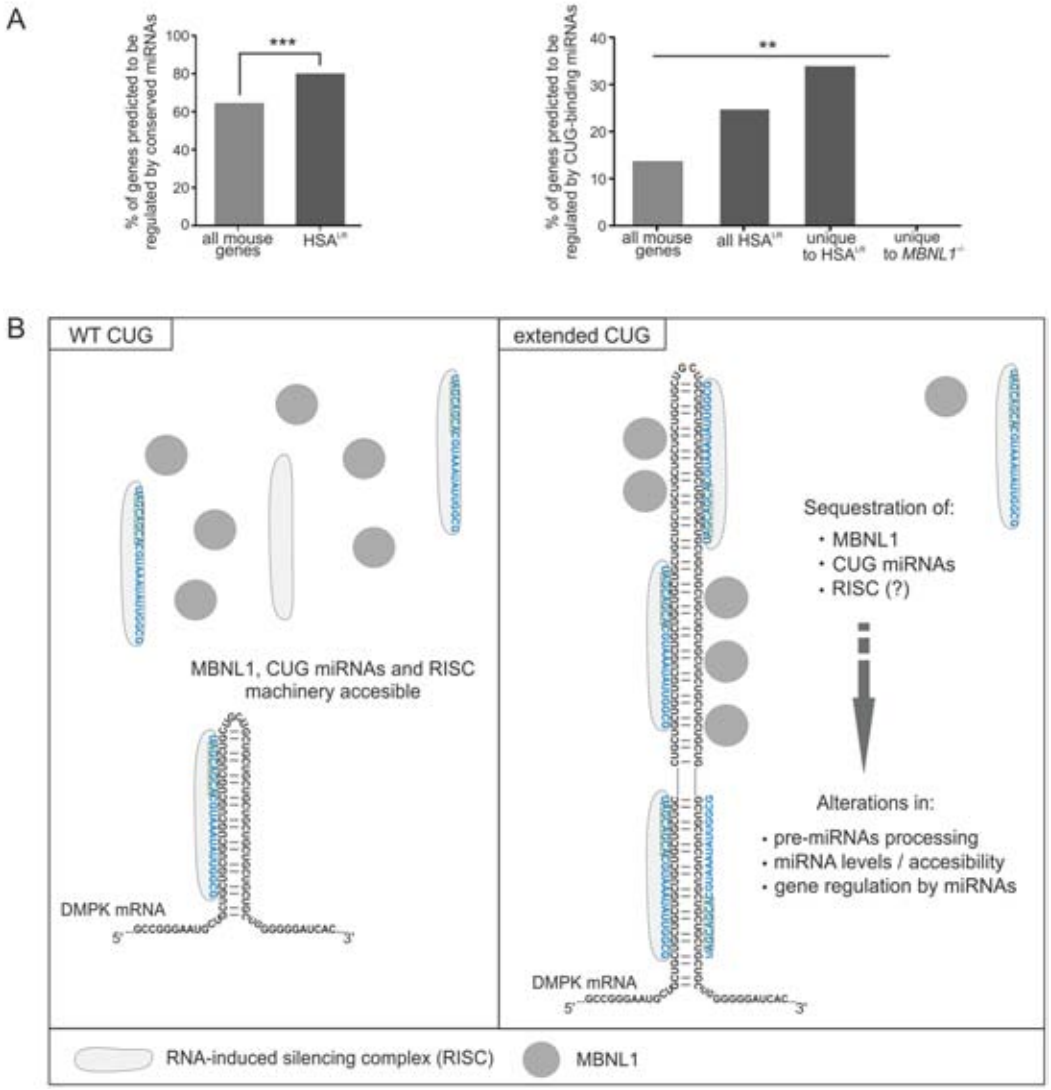

Figure 8. Potential sequestration of CUG-repeat binding miRNAs. (**A**) Deregulated genes in the CUG transgenic mice that may be subject to regulation by CUG-repeat-binding miRNAs (62). The left graph shows the percentage of genes bearing putative b.s. for the conserved miRNAs in all murine transcripts and the transcripts that are deregulated in the CUG transgenic mice (HSA^{LR}). The right graph compares the percentage of genes with the predicted miRNA b.s. for miRNAs with CAG sequences in their seed regions (Supplementary Table S5) in the following groups: all murine transcripts (all mouse genes), all transcripts deregulated in the CUG transgenic mice (all HSA^{LR}), the transcripts deregulated in CUG transgenic mice, but not in the *Mbnl1*$^{-/-}$ or *Clcn1*$^{-/-}$ mice (unique to HSA^{LR}), and the transcripts deregulated only in the *Mbnl1*$^{-/-}$ mice, but not in the CUG transgenic mice (unique to *Mbnl1*$^{-/-}$). All comparisons were made using Yates' continuity corrected chi-squared test. **$-P$-value < 0.01, ***$-P$-value < 0.001. (**B**) Effects of the extended CUG repeats on miRNA activity. The mutant DMPK transcripts may sequester miRNAs with CAG repeats in their seed sequence. The sequestration of miRNAs could prevent their normal activity, leading to the reduction of accessible miRNA molecules and, consequently, improper repression of their targets.

148a sites in the intact DMPK 3′ UTR measured between the 5′ ends of both seeds was 15 nt. Based on the knowledge that these two miRs may act on the DMPK mRNA, we analyzed the binding mechanism of the miR-206/148a pair. We found that this pair may cooperate because the joint binding of miRs 206 and 148a increased the down-regulation of DMPK expression. The reporter construct carrying the sequence corresponding to the native b.s. of miR-148a and miR-206 (WT_148/206) with the 5′ seed ends spaced by 15 nt (the lower limit of the cooperative distance) was efficiently suppressed by these miRNAs; and the suppression was greater than that achieved by either of the single miRNAs. Interestingly, Ext_148/206, the reporter construct with slightly extended miRNA sites (to 31 nt between the 5′ ends of the seed) showed stronger suppression (by approximately 20%). Of the three reporter constructs that were tested in our study, Ext_148/206 was the most effectively targeted construct by the simultaneously expressed miRs 148a and 206. The observed effect was consistent with previous findings showing that cooperativity is facilitated when the miRNA b.s. are directly adjoining (3,65) or are separated by a few additional nucleotides (66). In the case of the Long_148/206 construct with miRNA seed ends separated by 85 nt, the measured repression was restored to the

level of the WT reporter. A similar decrease in cooperative repression has been reported previously (5,65). It has also been assumed that the availability of miRNA sites that were separated is reduced due to the lower concentration of b.s. (25), and that the loss of the directly adjacent b.s. may lead to a shift toward independent activities (7). Together, our results indicate that miR-148a and miR-206 are functional when their b.s. are located at a distance between 15 and 85 nt, and this observation may relate to the regulation of other genes targeted by these miRNAs. Here, we refer to miRNA cooperativity because the level of joint repression produced by these two miRNA sites was stronger than the repression induced by only one of them; however, this repression was still not greater than that expected from the independent contribution of two single sites. In particular, the resulting increase in the level of suppression (by approximately 20%) resembled the effect obtained using multiple (but the same) rather than single binding sites in the reporter constructs (32).

Of particular importance is the fact that the studied miRNAs exhibit different expression profiles; miR-148a is ubiquitously expressed in many tissue and cell types, whereas miR-206 is almost exclusively expressed in muscles. The synergistic and/or antagonizing activity of miRNAs with different expression levels could be employed in therapeutic interventions using miRNA mimics and/or miRNA inhibitors. The natural selective activity of two miRNAs with overlapping binding sites has been previously reported (9). Our genome-wide search for pairs of miRNA sites based on their expression profiles suggests that both competition for binding sites and miRNA cooperativity by binding to adjacent sites are common miRNA regulatory mechanisms.

In summary, our findings for miRNA cooperativity provide mechanistic insights into the miRNA-mediated regulation of the DMPK transcript. Interference with miRNA function has known therapeutic potential; therefore, better knowledge of the miRNA-mediated gene regulation is essential because these potent regulatory RNAs can be used in various therapeutic strategies.

CUG repeats present in the DMPK 3′ UTR may sequester CUG-repeat-binding miRNAs

The presence of abnormally long CUG repeats in the DMPK 3′ UTR can potentially affect the general physiological miRNA-mediated regulatory mechanisms of gene expression because these expanded CUG repeats can be a target of the miRNA silencing machinery. Using bioinformatics, Hon *et al.* identified a group of 7 miRNAs that were predicted to bind to CUG repeats via the CAG sequences present in their seed regions. It was also calculated that the miRNA binding affinity to CUG repeats may increase with the number of CUG repeat sequences (25).

In the present study, we performed a detailed analysis of miRNA binding to CUG repeats in the DMPK 3′ UTR using both computational and experimental approaches. Using luciferase assays, we examined three miRNAs and demonstrated that miR-15b/16 can significantly down-regulate its target. Importantly, the repression efficiency of this miRNA increased with the length of the repeated CUG sequence. This result corroborates the findings that certain miRNAs directly target the expanded CUG repeats in the DMPK mRNA and that the degree of miRNA-mediated repression increases with the length of the repeated sequence. Because human miRNAs are able to decrease mRNA levels (42), mutant mRNAs with expanded CUG triplets may serve as targets for therapeutic intervention by reducing the amount of the toxic transcripts. Recently, several approaches aimed at targeting CUG repeats with antisense oligonucleotides and short interfering RNAs (siRNAs) have been proposed (67–70); therefore, close attention should also be paid to the potential use of natural or artificial miRNA binding to such repeats.

Having examined the miRNA targeting of the CUG repeat tracts of different lengths present in the DMPK transcript using the luciferase system, we examined the potential sequestration of these miRNAs by the exogenously expressed mutant DMPK mRNAs in cells. We overexpressed either CUG or CAG expanded repeats in the context of *DMPK* exons 11–15 (36) in HEK 293T cells and found that miR-16, with the 7-nt seed sequence AGCAGCA, was located in cytoplasmic foci formed by the mRNAs with CUG repeats. We propose that the mutant DMPK transcripts may serve as molecular sponges for natural miRNAs with CAG repeats in their seed sequences, sequestering them and thereby preventing their physiological activity. We speculate that in addition to the sequestration of specific RNA-binding proteins, such as the MBNL family proteins (12,13), the CUG repeats may also bind certain miRNAs and RNA-induced silencing complexes (RISCs).

The extension of the CUG repeats amplifies the naturally existing overlapping miRNA b.s., and our luciferase assay experiments showed that the miRNA binding capacity increases with the length of the CUG tracts, which may promote miRNA cooperativity. The mechanism of concerted miRNA regulation by two or more different miRNAs with overlapping or neighboring b.s. has not been fully elucidated, and we can only speculate how these miRNAs are associated with the CUG tracts. The footprint of argonaute (Ago), a central component of RISCs, was experimentally defined as 45–62 nt with the use of crosslinking immunoprecipitation of Ago protein-RNA complexes (71), which indicates that Ago bound to one b.s. covers the other sites positioned in close vicinity and prevents the binding of other Ago molecules. Although long tracts of triplet repeats are likely to form tight hairpin structures, the hairpin stems are easily accessible to RNA binding proteins (reviewed in (72)), and we think that they are likely to be the association sites for RISCs loaded with CUG binding miRNAs. However, we cannot excluded the possibility that miRNAs containing CAG sequences in their seed regions can directly associate with CUG repeats without being constantly associated with RISCs, because the presence of stable Ago-free miRNA–mRNA duplexes has been recently demonstrated in mammalian cells (73). The increase in the local concentration of CUG binding miRNAs associated with CUG tracts could then enhance the preference for Ago binding, resulting in sequestration of the miRNA silencing machinery. In this context, the sequestration of CUG-repeat-binding miRNAs could cause them to become inactive or simply prevent them from regulating other transcripts that contain these miRNA b.s., leading to a more widespread

impairment of the miRNA-mediated regulation of gene expression (Figure 8B).

It has been demonstrated that CTG expansions are directly linked to alterations in miRNA regulation and RNA toxicity in DM1 (24). An analysis of the muscle miRNA transcriptome in a *Drosophila* model of CUG toxicity revealed 20 alterations in the miRNA profiles triggered by the expression of repeats, and the misregulation of several miRNAs has been shown to be independent of Mbnl1 sequestration. In addition, the expansion of CUGs in transgenic mice has been shown to affect gene expression at the RNA level, and the observed effects were mediated mainly, but not entirely, through the sequestration of Mbnl1 (62).

We consider that the sequestration of the CUG-repeat-binding miRNAs may have wider implications for the RNA-mediated regulation of gene expression. Long noncoding RNAs (lncRNAs) were recently shown to play a vital role in connecting many RNA regulatory pathways (74–78). A growing number of direct links between the levels of miRNAs, mRNAs and lncRNAs are being discovered (recently reviewed in (79), and lncRNAs are thought to act as competing endogenous RNAs (ceRNAs) and miRNA sponges (80)). Based on these reports, we speculate that the sequestration of CUG-repeat-binding miRNAs can affect the expression levels of lncRNAs with binding sites for these miRNAs. Imbalances between miRNAs and interacting ceRNAs due to altered expression levels or the cellular distribution and mutagenesis of interacting sites have been shown to contribute to disease pathogenesis (80). It was also proposed that competitor RNAs should be among the most abundant RNAs in the cell, or that they should contain dozens of binding sites for a single miRNA species (81,82). In this scenario, the long CUG repeats extended in DM1 appear to be ideal candidates. Therefore, we believe that lncRNAs may play a role in the pathogenesis of TREDs, and future studies are needed to unravel their functions.

In conclusion, it is of utmost importance to understand the role of miRNAs in DM1 to identify potential novel therapies. Given the complexity and degree of interactions between miRNAs and their various targets, which may be either protein-coding or non-coding transcripts, a better understanding of the rules governing gene regulation by noncoding RNAs (ncRNAs) and the intricate mechanisms that are involved in DM1 pathogenesis is necessary.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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