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Expanded CTG repeats trigger miRNA alterations in *Drosophila* that are conserved in myotonic dystrophy type 1 patients

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Myotonic dystrophy type 1 (DM1) is caused by the expansion of CTG repeats in the 3′ untranslated region of the *DMPK* gene. Several missplicing events and transcriptional alterations have been described in DM1 patients. A large number of these defects have been reproduced in animal models expressing CTG repeats alone. Recent studies have also reported miRNA dysregulation in DM1 patients. In this work, a *Drosophila* model was used to investigate miRNA transcriptome alterations in the muscle, specifically triggered by CTG expansions. Twenty miRNAs were differentially expressed in CTG-expressing flies. Of these, 19 were down-regulated, whereas 1 was up-regulated. This trend was confirmed for those miRNAs conserved between *Drosophila* and humans (*miR-1*, *miR-7* and *miR-10*) in muscle biopsies from DM1 patients. Consistently, at least seven target transcripts of these miRNAs were up-regulated in DM1 skeletal muscles. The mechanisms involved in dysregulation of *miR-7* included a reduction of its primary precursor both in CTG-expressing flies and in DM1 patients. Additionally, a regulatory role for Muscleblind (Mbl) was also suggested for *miR-1* and *miR-7*, as these miRNAs were down-regulated in flies where Mbl had been silenced. Finally, the physiological relevance of miRNA dysregulation was demonstrated for *miR-10*, since overexpression of this miRNA in *Drosophila* extended the lifespan of CTG-expressing flies. Taken together, our results contribute to our understanding of the origin and the role of miRNA alterations in DM1.

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is a chronic, slowly progressing multisystemic disease, with symptoms that include loss of muscle strength, myotonia and excessive fatigue (OMIM #160900). DM1 is caused by a dynamic expansion of CTG repeats in the 3′ untranslated region of the *dystrophia*

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myotonica-protein kinase (*DMPK*) gene (for a recent review, see 1). Characteristic molecular features of the disease have been associated with a toxic RNA gain of function of the CUG expansions. Expanded CUG repeats have been demonstrated to be toxic *per se* in several cell types and animal models (2–4), disrupting transcription and alternative splicing of at least 175 genes and 156 defined pre-mRNAs in mice, respectively (5). Expanded CUG repeats sequester nuclear proteins and accumulate into distinctive foci within muscle and neuronal nuclei (6–8). The alternative splicing factor Muscleblind-like 1 (MBNL1) is recruited into these foci, causing loss-of-function of the protein, which has been linked to critical DM1 features (5,9). An antagonistic splicing factor of MBNL1 activity, CUGBP Elav-like family member 1 (CELF1), is hyper-phosphorylated and subsequently stabilized in DM1 (10). Despite its ability to bind to CUG triplets, CELF1 is not sequestered into CUG-RNA foci (11,12). Instead, CELF1-mediated alterations in DM1 models require the presence of *DMPK* (11,13). Together, MBNL1 and CELF1 regulate critical alternative splicing transitions during heart and skeletal muscle development, which are dysregulated in DM1 (14–16).

In addition to alternative splicing, several other mechanisms have been recently found, which add complexity to the molecular pathology of DM1. These include repeat-associated non-ATG translation (RAN) (17), bidirectional transcription (18), aberrant DNA methylation (19,20) or microRNA (miRNA) dysregulation, among others (21,22). miRNAs are short non-coding RNAs present in all eukaryotes, which regulate gene expression by decreasing their target mRNA levels, or by blocking their translation (23,24). miRNA biogenesis is well understood. In the canonical biogenesis, miRNAs are produced from long primary transcripts (pri-miRNA) that are first processed by Drosha in the nucleus to generate one or more hairpin structures known as pre-miRNA. Then, Dicer orchestrates another cut to generate the mature miRNA in the cytoplasm (Supplementary Material, Fig. S1). Computational methods have predicted that miRNAs account for ~1% of all eukaryotic genes, and that one-third of the transcriptome may be regulated by miRNAs (25). In animals, miRNAs participate in virtually all cell functions, including the regulation of differentiation, proliferation and apoptosis. A subset of miRNAs known as myomiRs are highly enriched in heart and skeletal muscles, where they can regulate muscle function in development and disease (26–28). Eisenberg *et al*. (29) described an miRNA expression profile from 10 human muscle disorders and identified 185 miRNAs that were dysregulated in almost all cases, further demonstrating the importance of miRNAs in muscle function. In DM1, some studies using muscle biopsies have described that *miR-206*, *miR-210*, *miR-1* and *miR-335* are over-expressed in skeletal muscle, whereas *miR-29b*, *miR-29*c and *miR-33* are down-regulated. However, independent studies have found either no changes in *miR-1* levels or a reduction of this miRNA, as well as an altered cellular distribution of muscle-specific *miR-1*, *miR-133b* and *miR-206* (21,22,30).

Given that the origin of miRNA alterations in DM1 remains uncertain, in this work we have used a *Drosophila* model expressing CTG repeats in the absence of an ATG start codon, in order to study the contribution of CTG expansions to miRNA defects. We have found that the expression of 20 miRNAs is affected by expression of CTG repeats in DM1 flies. Dysregulation of conserved miRNAs *miR-1*, *miR-7* and *miR-10a* also occurred in DM1 patients, where a number of target transcripts were consistently up-regulated. For some of these miRNAs, reduced levels originated from down-regulation of their pri-miRNA precursors and/or Muscleblind loss-of-function. Importantly, over-expression of *miR-10* in *Drosophila* extended the lifespan of model flies. All together, these results contribute to our understanding of the nature and pathological implications of miRNA dysregulation in DM1.

RESULTS

Expression of expanded CTG repeats in *Drosophila* causes reduction of defined miRNAs

An increasing number of miRNAs have been found altered in DM1 patients, where CTG repeats are present in the context of full-length *DMPK* transcripts. In order to identify miRNA alterations directly caused by CTG expansions, we targeted the expression of 480 interrupted CTG repeats, *UAS-(iCTG)480*, to the *Drosophila* muscles with the *Myosin heavy chain (Mhc)-Gal4* driver line. We obtained the miRNA transcriptome (miRNome) profile of two independent i(CTG)480 transgenic lines (*Mhc-Gal4>UAS-i(CTG)480 1.1* and *Mhc-Gal4>UAS-i(CTG)480 2.2*), using SOLiD^{TM} 3 sequencing of small-RNA libraries. Small-RNA libraries from *Mhc-gal4/+* flies were used as controls. Statistical analysis revealed 20 miRNAs differentially expressed in DM1 flies *versus* control individuals (Fig. 1A and Supplementary Material, Table S1). Nineteen of these were down-regulated in the presence of CTG expansions, whereas one was up-regulated.

Among the 20 miRNAs altered in DM1 flies, we chose to validate the effect of CTG repeats on *miR-1*, *miR-7* (given their conservation in humans) and on *miR-1003* (given its miRtronic nature) by northern blot of small RNAs, using LNA probes and normalizing against the endogenous small nuclear RNA (snRNA) U6. Consistent with our SOLiD^{TM} 3 sequencing data, northern blot analysis revealed expression values for *miR-1*, *miR-7* and *miR-1003* that were reduced by 30% ($P = 0.0047$), 50% ($P = 0.0052$) and 30% ($P = 0.0384$), respectively, compared with control flies (Fig. 1B and C). Therefore, these results demonstrate that expanded CTG-repeat expression affects the *Drosophila* miRNome mainly by causing a reduction in the level of specific miRNAs.

CTG-induced down-regulation of *miR-1*, *miR-7* and *miR-10* is conserved between *Drosophila* and DM1 patients

Among the 20 miRNAs that we found altered in CTG-expressing flies, *miR-1*, *miR-7* and *miR-10* were conserved between *Drosophila* and humans (Supplementary Material, Fig. S2). In order to validate the relevance of our findings in DM1, we used qRT–PCR to study the levels of human *miR-1*, *miR-7*, and *miR-10* (*miR-10a* and *miR-10b*) in

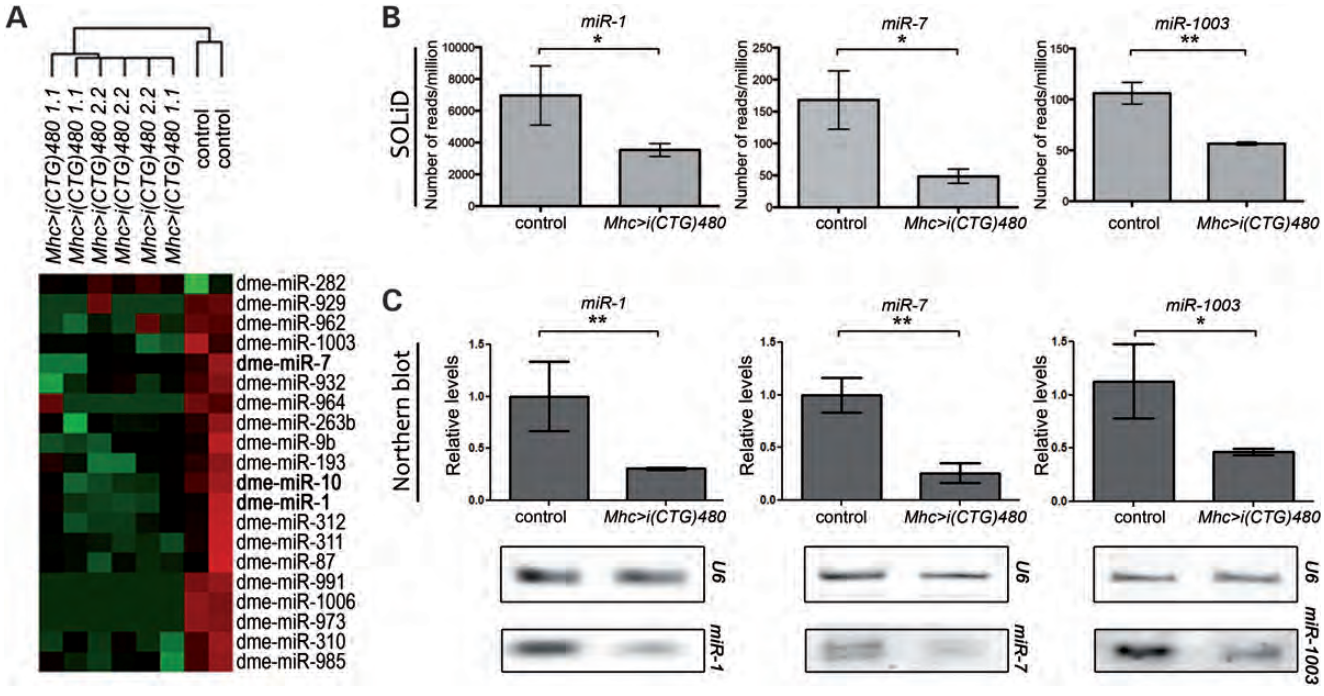

Figure 1. Expression of expanded CTG repeats in the *Drosophila* muscles reduced the levels of specific miRNAs. (**A**) Heat map graphical representation and clustering analysis of miRNA expression from two independent *UAS-i(CTG)480* lines (*Mhc-Gal4>UAS-i(CTG)480 1.1* and *Mhc-Gal4>UAS-i(CTG)480 2.2*) and control flies (*Mhc-Gal4/+*). Two biological replicates of each genotype ($n = 50$ per replicate) and two technical replicates were analyzed (Supplementary Material, Table S1). Data are presented as a dendrogram, with the closest branches of the tree showing samples with less dissimilar expression patterns. Green and red indicate statistically significant down- and up-regulated miRNAs compared with controls, respectively (ANOVA, $\alpha = 0.05$). These miRNAs represented ~13% of the *Drosophila* miRNome at the time the SOLiD data were processed (miRBase release 13.0; containing 152 miRNAs). Conserved miRNAs between *Drosophila* and humans appear in bold font. (**B**) Graphic representation of the *miR-1*, *miR-7* and *miR-1003* expression data from SOLiD™ 3 sequencing showing a similar reduction in miRNA levels from *Mhc-Gal4>i(CTG)480* flies compared with *Mhc-Gal4/+* individuals. Expression levels are given as number of reads per million. (**C**) Validation of the results shown in (B) by northern blot of small RNAs and densitometry analysis of the bands showing a reduction for *miR-1*, *miR-7* and *miR-1003*, respectively. Endogenous U6 was used for normalization in northern blot experiments, and miRNA expression levels are shown relative to control flies. Four biological replicates ($n = 50$ per replicate) and two technical replicates per biological sample were analyzed. All graph bars represent average fold changes and their standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$.

skeletal muscle biopsies (biceps, vastus and deltoid) from five DM1 patients (aged 47 ± 5) compared with biopsies from three healthy individuals (aged 58 ± 11) and normalized against endogenous snoRNA U48. Statistical analysis revealed a significant reduction in the levels of *miR-1* (72%; $P = 0.0022$), *miR-7* (80%; $P = 0.0004$) and *miR-10a* (60%; 0.0271) in DM1 patients compared with control individuals (Fig. 2A). *miR-10b*, which is less similar to *Drosophila miR-10* than human *miR-10a*, showed a non-significant increase. Therefore, these results demonstrate the conservation of miRNA dysregulation triggered by expanded CTG repeats between our i(CTG)480 *Drosophila* model and humans. Interestingly, *miR-1* levels had been previously studied in DM1 patients by independent groups, which found either no changes in *miR-1* levels (22), a 2.1-fold reduction (21), or a 1.9-fold up-regulation (30); whereas *miR-7* and *miR-10* had not been tested, nor associated with the disease before.

To investigate whether *miR-1*, *miR-7* and *miR-10a* dysregulation had an effect on their mRNA targets, we used gene microarray data from muscle biopsies of DM1 patients previously generated in our laboratory, where a number of misexpressed genes had been identified (unpublished data). Of all Targetscan (25) predictions that were represented among these genes, we found 21 *miR-1* targets, 14 *miR-7* targets and 7 *miR-10* targets. All of them but one were significantly up-regulated (>10-fold) in DM1 muscle biopsies (Supplementary Material, Table S2 and Fig. 2B; upper graphs). However, the target transcripts of three randomly selected control miRNAs (*let7a*, *miR-340* and *miR-454*) showed changes in their expression levels that indistinctly included up- and down-regulation, ruling out an miRNA-mediated effect, and further supporting the specificity of our results (Fig. 2B; lower graphs). Among the total of up-regulated targets of *miR-1*, *miR-7* and *miR-10*, a subset of 13 were chosen for their validation by qRT–PCR from muscle biopsies of six DM1 patients. An apparent up-regulation of mRNA levels was observed in each case, although only seven of these changes were statistically significant (Fig. 2C–E; Supplementary Material, Table S3). Confirmed genes encoded proteins that participated in a mixed range of cellular processes, including antioxidant enzyme SOD1, transcriptional regulator SMARCA4 and nucleotide exchange factor NET1 (*miR-1*; Fig. 2C); amyloid precursor protein secretase CTSB, autophagy regulator ATG4 or cytoskeletal protein VCL (*miR-7*; Fig. 2D); and the member of the SUMOylation

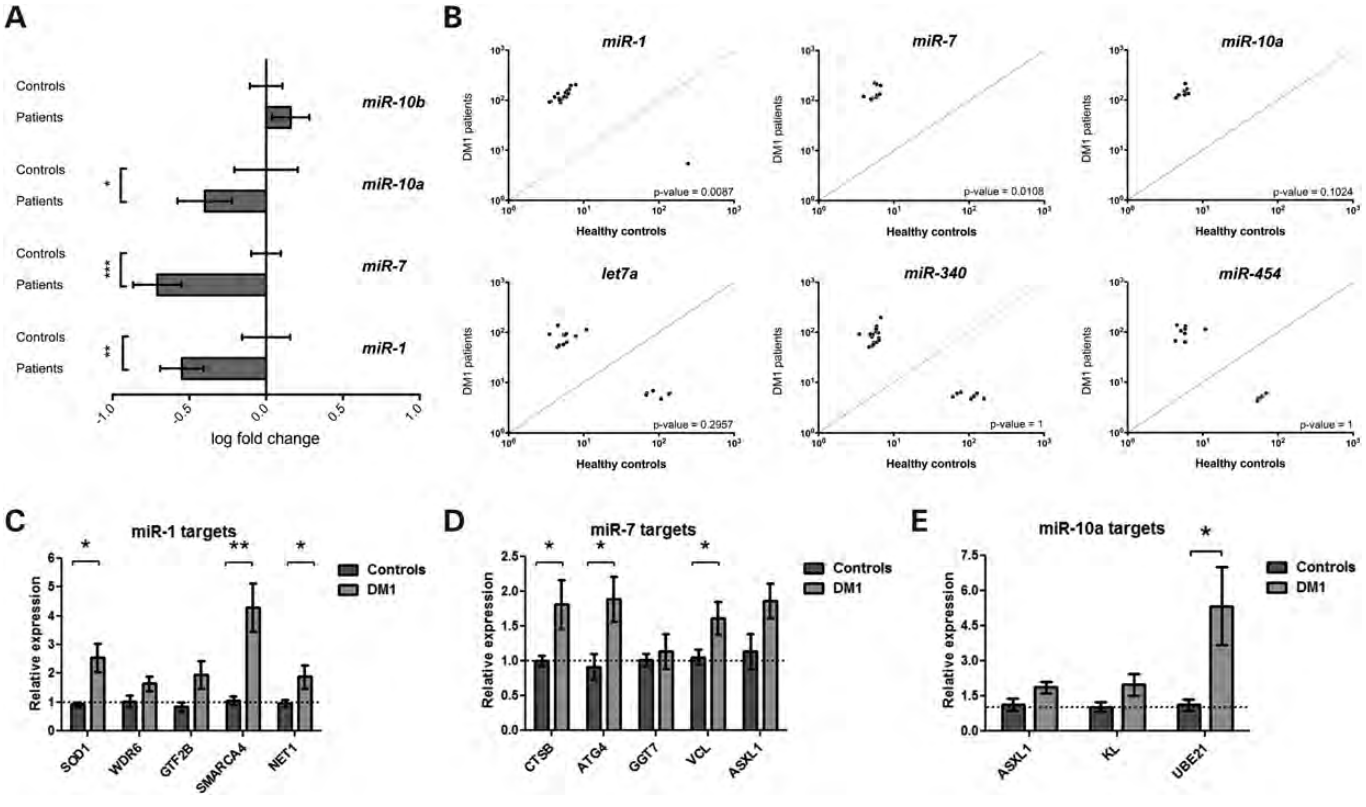

Figure 2. miRNA alterations are conserved between *Drosophila* and DM1 patients. (**A**) miRNA log fold change values of *miR-1*, *miR-7*, *miR-10a* and *miR-10b* based on qRT–PCR expression data from adult skeletal muscle biopsies of five DM1 patients and three healthy individuals (control). Graph bars represent average fold changes of miRNA expression in logarithmic scale, calculated by the $2^{-\Delta\Delta C_t}$ method, and their confidence intervals. sno-RNA RNU48 was used as the endogenous control. (**B**) Scattered plot representation of the microarray signal values of the altered transcripts predicted to be regulated by *miR-1*, *miR-7* and *miR-10a* (Targetscan; upper row). Altered transcript targets of a subset of three randomly chosen miRNAs (*let7a*, *miR-340* and *miR-454*) are also shown (lower row). Consistent with a decrease in *miR-1*, *miR-7* and *miR-10a*, an up-regulation of their predicted targets was observed, whereas both up- and down-regulations were detected for *let7a*, *miR-340* and *miR-454* (Fisher's exact test, $\alpha = 0.05$). (**C**–**E**) qRT–PCR validation analysis from adult skeletal muscle biopsies of six DM1 patients and six healthy (control) biopsies. The expression levels of 13 selected *miR-1* (C), *miR-7* (D) and *miR-10a* (E) targets are shown relative to the control individuals. An up-regulation trend was observed in all cases, although only the indicated seven were statistically significant. In this case, *GADPH* was used as the endogenous control. Graph bars represent average fold changes of gene expression, calculated by the $2^{-\Delta\Delta C_t}$ method, and their standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

machinery UBE21 (*miR-10*; Fig. 2E). Thus, our results suggest a wide pathologic potential of miRNA dysregulation in DM1.

Finally, to assess whether *miR-1*, *miR-7* and *miR-10* alterations originated during muscle differentiation, we also studied the levels of *miR-1*, *miR-7*, *miR-10a* and *miR-10b* in control and DM1-derived human skin fibroblasts before and after they had been induced to turn on the myogenic program by expression of the murine MyoD (31). Before myogenesis was induced, the levels of *miR-1* or *miR-10b* did not differ between control and DM1 fibroblasts ($P = 0.6534$ and $P = 0.8049$, respectively); whereas *miR-10a* was slightly increased ($P = 0.0048$), and *miR-7* was notably reduced in DM1 fibroblasts compared with controls ($P = 0.0009$) (Fig. 3A; Supplementary Material, Table S4). After the induction of myogenesis, however, all four miRNAs were significantly reduced in DM1 cells (*miR-1*, $P = 0.0248$; *miR-7*, $P = 0.0312$; *miR-10a*, $P = 0.0002$; and *miR-10b*, $P = 0.0166$). Interestingly, for *miR-7*, this reduction was less marked in muscle cells than in fibroblasts (Fig. 3B and Supplementary Material, Table S4). These results suggest that different miRNA alterations in DM1 muscle cells may originate from different mechanisms.

Expanded CTG repeats alter the levels of defined pri-miRNA precursors

SOLID^{TM} 3 sequencing identified *Drosophila miR-310*, *miR-311* and *miR-312* among the initial 19 miRNAs that were reduced in CTG-expressing flies. Interestingly, these miRNAs formed an miRNA cluster (i.e. a group of miRNAs contained within the same gene) (32) denominated cluster *miR-310–313*, which is transcribed as a polycistronic primary precursor. *miR-313*, which completes the cluster, showed a similar trend, although its reduction was not statistically significant (Fig. 4A and Supplementary Material, Table S5). *miR-960*, *miR-962* and *miR-964*, which were also altered in DM1 flies, were part of a second miRNA cluster (cluster *miR-959–964*) located in the antisense (−) strand of the *CG31646* gene. *miR-959*, *miR-961* and *miR-963*, the rest of the members of this cluster, also showed a mild reduction in CTG-expressing flies (Fig. 4B and Supplementary Material,

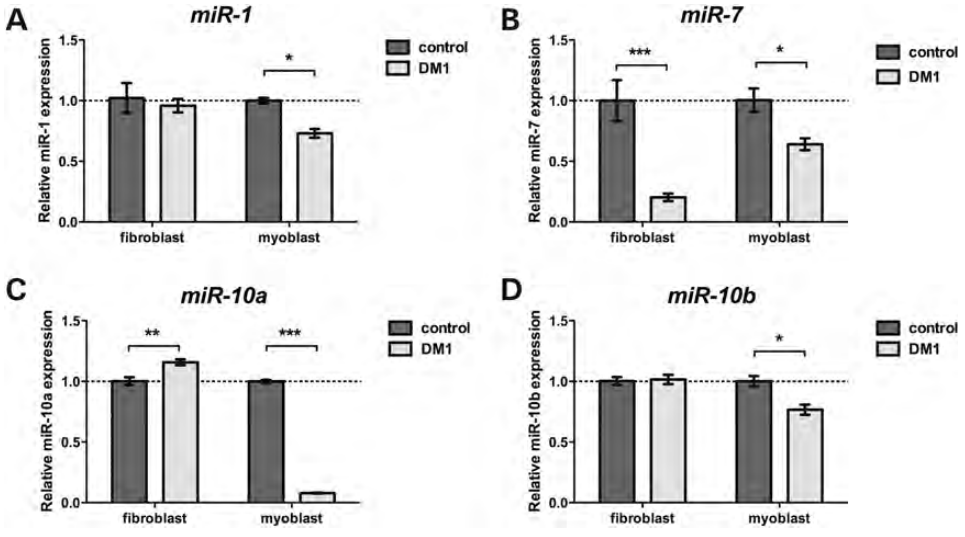

Figure 3. *miR-1*, *miR-7* and *miR-10* are down-regulated in DM1 cells. (**A**–**D**) qRT–PCR amplification of mature *miR-1*, *miR-7*, *miR-10a* and *miR-10b* miRNAs from healthy and DM1 patient-derived fibroblasts (having 333 CTGs at the time of diagnosis, in 2010). miRNA levels were measured before and after cell induction to activate myogenic transdifferentiation (MT). *miR-7* was reduced in both DM1 fibroblasts and DM1 transdifferentiated myoblasts (B), whereas *miR-1* (A), *miR-10a* (C) and miR-*10b* (D) were down-regulated only after the myogenic program had been activated in DM1 cells. *snoRNA U48* was used as the endogenous gene, and all data were normalized relative to the control group. Two biological samples and three technical replicates per biological sample were used. All graph bars represent average fold changes of miRNA expression, calculated by the $2^{-\Delta\Delta C_t}$ method, and their standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Additional statistical data are shown in Supplementary Material, Table S4.

Table S5). Therefore, these results suggested that the changes in the expression levels of these miRNAs could originate from a reduction in their pri-miRNAs. To confirm this hypothesis, we measured the pri-miRNA levels of both clusters by qRT–PCR from *Mhc-Gal4>UAS-i(CTG)480* and *Mhc-gal4/+* (control) flies (Fig. 4D and E). *Mhc-Gal4>UAS-i(CTG)480* flies showed a significant reduction in the pri-miRNA levels of *miR-310–313* ($P = 0.0077$) and *miR-959–964* ($P = 0.0368$) clusters when compared with controls, demonstrating a CTG-mediated effect on both transcription units. We also measured the levels of sense (+) strand mRNA from the *CG31646* gene and found no significant changes compared with controls ($P = 0.5330$), ruling out a non-specific effect on *miR-959–964* caused by a reduction of the expression of this gene (Fig. 4E). The pri-miRNA levels of the cluster *miR-2a-1–2b-2*, which encodes miRNAs that were not modified by CTG expression in our SOLiD^{TM} 3 analysis, were also studied. No significant differences in the pri-miRNA of this cluster were detected in CTG-expressing flies when compared with controls (Fig. 4C and F; $P = 0.2368$), further confirming the specificity of the effect of CTG repeats on pri-miRNA production of defined miRNAs. Molecular mapping of the i(CTG)480 1.1 and 2.2 insertions used in this study showed no proximity to the *miR-310–313* or *miR-959–964* clusters, excluding the possibility of a transcriptional repression caused by CTG-induced alterations of the local chromatin structure (Supplementary Material, Table S6).

Based on these findings, we next investigated whether the pri-miRNA levels of conserved miRNAs *miR-1*, *miR-7* and *miR-10* could also be affected by expression of expanded CTG repeats. In *Drosophila*, qRT–PCR analysis revealed that the pri-miRNAs of *miR-1* and *miR-10* were comparable with control flies ($P = 0.3435$ and 0.7095, respectively). However, the pri-miRNA of *miR-7* was significantly reduced (Fig. 4G; $P = 0.0420$). This effect was also observed in skeletal muscle biopsies from six DM1 patients, where the pri-miRNA levels from human *miR-7* genes *pri-miR-7-1* and *pri-miR-7-2*, but not *pri-miR-7-3*, were significantly reduced compared with healthy controls ($P = 0.0339$, $P = 0.0209$ and $P = 0.0917$, respectively, Fig. 4H). Therefore, these results demonstrate that, at least partly, *miR-7* is reduced in *Drosophila* and in DM1 patients due to a down-regulation of its pri-miRNA precursor.

Muscleblind is necessary for the regulation of *miR-1* and *miR-7* in *Drosophila*

In addition to the down-regulation of specific primary precursors, changes in miRNA levels may also occur at a downstream level, including the regulation of their biogenesis or their stability. Human MBNL1 has been described to participate in the biogenesis of *miR-1* (21) by binding to a UGC motif located within its pre-miRNA, favoring the generation of mature *miR-1*. To confirm whether the *Drosophila* homolog of MBNL1 (Mbl) was also involved in the differential expression of *miR-1* in *Drosophila*, we used transgenic flies carrying an RNAi construct targeted against all Mbl isoforms (*UAS-IR-mbl*). In a wild-type background, *mbl* silencing using an *Mhc-Gal4* driver line (*Mhc-Gal4>UAS-IR-mbl*) caused a significant reduction of *miR-1* levels as detected by northern blot ($P = 0.0102$). On the contrary, over-expression of MblC isoform did not induce changes in *miR-1* (Fig. 5A and B; Supplementary Material, Fig 3A). This indicates that (i) other Mbl isoforms may also be important in regulating the levels of *miR-1*, or (ii) that the amount of miRNA

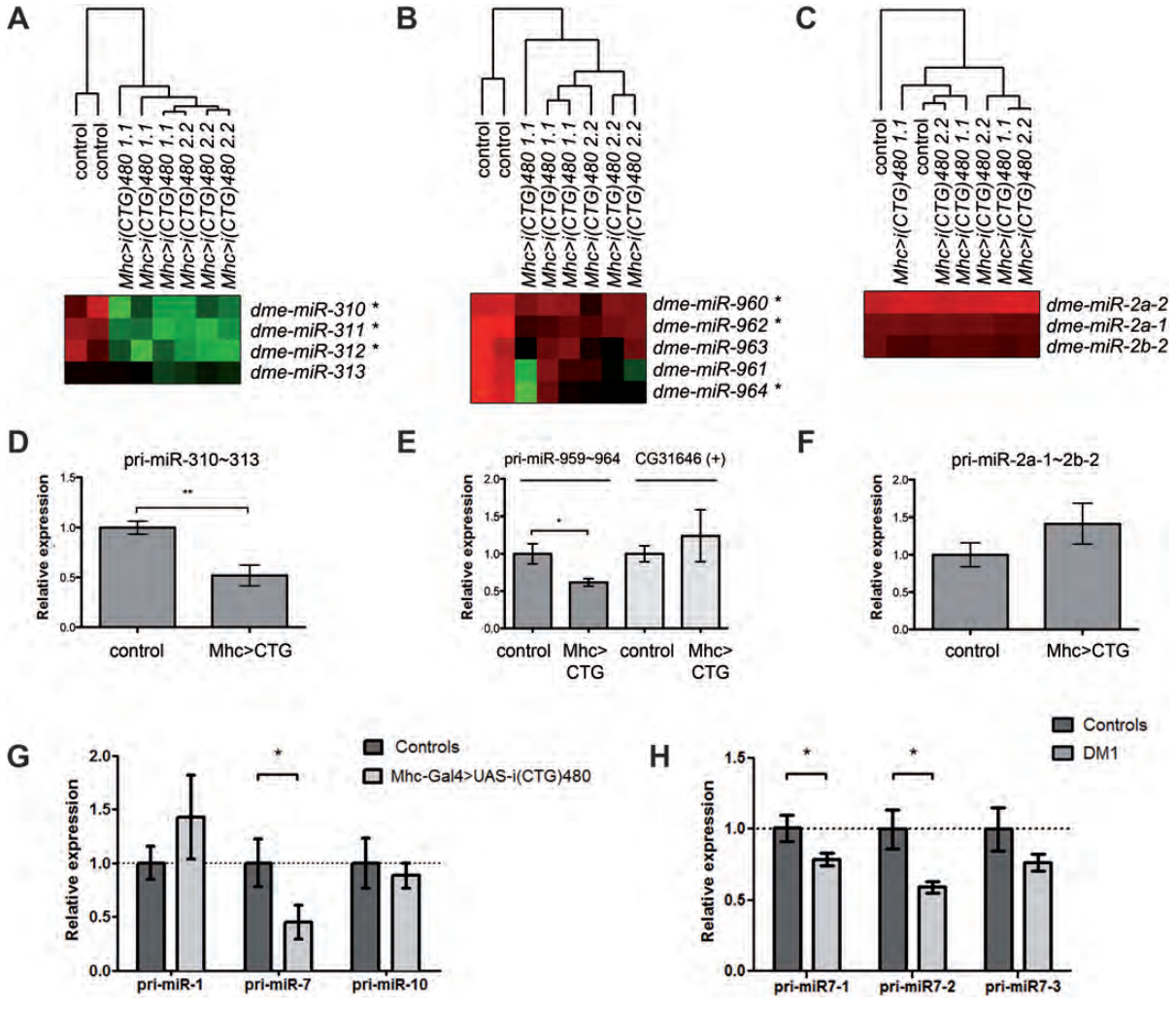

Figure 4. Expanded CTG repeats cause down-regulation of specific pri-miRNAs. (**A**–**C**) Heat map graphical representation of expression levels of clusters *miR-310–313* (A), *miR-959–964* (B) and *miR-2a-1–2b-2* (C). Hierarchical clustering analysis revealed significantly reduced levels of miRNAs from clusters *miR-310–313* (A) and *miR-959–964* (B) in CTG-expressing flies (*Mhc-Gal4>UAS-i(CTG)480 1.1* and *2.2*) compared with controls (*Mhc-Gal4/+*). No differences between groups were detected for the miRNA cluster *miR-2a-1–2b-2*, which was used as a negative control (C). (**D**–**F**) qRT–PCR amplification of the pri-miRNAs from clusters *miR-310–313*, *miR-959–964* and *miR-2a-1–2b-2* from adult flies confirmed a CTG-dependent reduction in the expression levels of the pri-miRNAs of clusters *miR-310–313* (D) and *miR-959–964* (E), but not *miR-2a-1–2b-2* (F), when compared with controls. The strand of the *CG31646* gene opposite to the strand that contains the cluster *miR-959–964* was used as an additional control, for which no expression differences were observed (E). (**G**) The pri-miRNAs of conserved miRNAs *miR-1*, *miR-7* and *miR-10* were also measured in *Drosophila* by qRT–PCR. Only the pri-miRNA of *miR-7* was significantly reduced in CTG-expressing flies. In (D–G), four biological replicates ($n = 50$ per replicate), and three technical replicates per biological sample, were used. Biological samples included two female groups and two male groups, and normalization was carried out relative to the corresponding gender controls. *tubulin84B* was used as the endogenous gene. (**H**) In human skeletal muscle biopsies, qRT–PCR revealed that the pri-miRNAs from two of the three genes encoding human *miR-7* were down-regulated in DM1 patients ($n = 6$ individuals; three technical replicates per individual) compared with healthy individuals ($n = 6$ individuals; three technical replicates per individual). In this case, *GADPH* was used as the endogenous gene. All graph bars represent average fold changes of gene expression, calculated by the $2^{-\Delta\Delta C_t}$ method, and their standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$.

precursor available might be the limiting factor during biogenesis, with endogenous Mbl levels being sufficient to saturate the process. Supporting the second hypothesis, *mblC* over-expression significantly rescued *miR-1* levels in DM1 flies, where endogenous Mbl function is compromised (Fig. 5A and B; $P = 0.0435$). *mbl* silencing in a wild-type background also reduced *miR-7* levels, although this change was not statistically significant ($P = 0.1978$). However, in this case, *mblC* over-expression did not affect this miRNA even in the presence of CTG repeats (Fig. 5C and D and Supplementary Material, Fig. S3B; $P = 0.7751$). Therefore, the relative contribution of Mbl to the generation of *miR-1* and *miR-7* seems to be different; and additional factors that are independent of Mbl would participate in the regulation of *miR-7*.

Interestingly, two of the miRNAs that we found down-regulated in *Drosophila*, *miR-1003* and *miR-1006*, were miRtrons (i.e. miRNAs that arise from spliced-out introns). Given that splicing alterations are a hallmark of DM1, we also analyzed the splicing of the introns that host *miR-1003* and *miR-1006* (intron 6 of the *CG6695* gene, and intron 4 of the *VhaSFD* gene, respectively). Analysis of *miR-1004*, an miRtron not found altered in CTG-expressing flies, was also

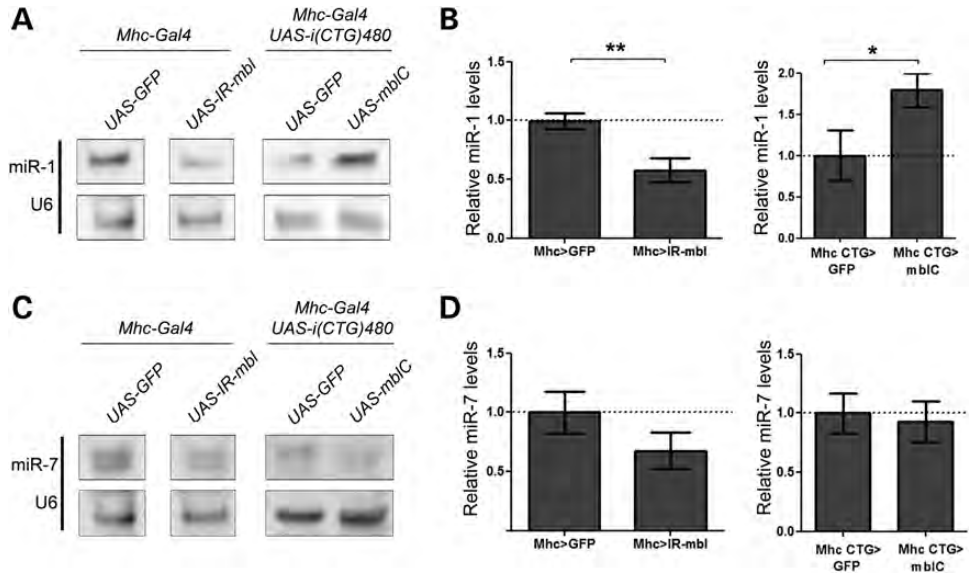

Figure 5. *Drosophila* Mbl regulates the levels of *miR-1* and *miR-7*. (**A**) Detection of *miR-1* by northern blot from adult fly males of the indicated genotypes, using LNA probes and densitometric quantification of the bands. (**B**) *mbl* silencing (*Mhc-Gal4>UAS-IR-mbl*) caused a 48% reduction in *miR-1* levels compared with control flies (*Mhc-Gal4>UAS-GFP*) (left). Co-expression of MblC and i(CTG)480 (*Mhc-Gal4 UAS-i(CTG)480>UAS-MblC*) resulted in a 55% rescue of the CTG-induced *miR-1* reduction when compared with flies co-expressing GFP and i(CTG)480 (*Mhc-Gal4 UAS-i(CTG)480>UAS-GFP*) (right). (**C**–**D**) *mbl* silencing also caused a 33% reduction of *miR-7* compared with control flies (left). However, MblC over-expression did not suppress the CTG-induced effect on *miR-7* levels (right). Endogenous U6 was used for normalization, and miRNA expression levels are shown relative to control flies. For each genotype, four biological replicates (50 individuals) and two technical replicates per biological sample were analyzed. All graph bars show average values and their standard errors. $^{*}P < 0.05$, $^{**}P < 0.01$.

included as a control. As expected, the splicing of intron 19 of the *CG43707* gene (*miR-1004* precursor) was unaffected (Supplementary Material, Fig. S4A and B). We did not detect *VhaSFD* transcripts containing intron 4 (*miR-1006* precursor), either in control or in DM1 flies (Supplementary Material, Fig. S4A), suggesting that this intron is completely spliced out in both cases. However, the levels of *CG6695* transcripts containing intron 6 (*miR-1003* precursor) were reduced by 24% in CTG-expressing flies compared with controls ($P = 0.0080$; Supplementary Material, Fig. S4A and B), indicating that intron 6 is spliced out at higher levels in DM1 flies than in controls. Taken together, the reduced levels of mature *miR-1003* and *miR-1006* in CTG-expressing flies cannot be explained by reduced splicing of their host transcripts, and would instead occur at a more downstream level.

Over-expression of *miR-10*, but not *miR-1* or *miR-7*, increases lifespan in CTG-expressing flies

To date, the pathogenicity of miRNA alterations in DM1 remains unclear. We studied the physiological relevance of CTG-induced miRNA down-regulation by performing a series of phenotypic rescue experiments using our DM1 *Drosophila* model, where we over-expressed *miR-1*, *miR-7* or *miR-10* under the control of the *Mhc-Gal4* driver line. We previously described that flies expressing i(CTG)480 in the musculature had a reduced lifespan compared with control individuals (2). Here, *Mhc-Gal4 UAS-i(CTG)480>UAS-GFP* flies showed a median survival (MS) of 15 days at 25°C, whereas control flies (*Mhc-Gal4>UAS-GFP*) showed an MS of 33 days at the same temperature (Fig. 6; $P < 0.0001$). Flies co-expressing i(CTG)480 and *miR-1* (*Mhc-Gal4 UAS-i(CTG)480>UAS-miR-1*) showed an intriguingly reduced MS (10 days; $P < 0.0001$) compared with CTG-expressing flies (Fig. 6A and D). This effect was not due to the toxicity of *miR-1*, as flies over-expressing *miR-1* alone (*Mhc-Gal4>UAS-miR-1*) showed a lifespan comparable with *Mhc-Gal4>UAS-GFP* controls (Fig. 6A).

The survival curves of flies co-expressing i(CTG)480 and *miR-7* (*Mhc-Gal4 UAS-i(CTG)480>UAS-miR-7*) also showed a mild but significant reduction in lifespan (MS of 11.5 days) compared with their controls, which co-expressed i(CTG)480 and dsRED (*Mhc-Gal4 UAS-i(CTG)480>-UAS-dsRED;* MS of 13 days) (Fig. 6B and D; $P = 0.0109$). In this case, this effect could be explained by the toxicity of *miR-7*, as over-expression of *miR-7* alone (*Mhc-Gal4>-UAS-miR-7*) resulted in an MS of 27 days, significantly lower than that of individuals expressing dsRED (*Mhc-Gal4>UAS-dsRED;* MS of 29.5 days; $P = 0.0310$) (Fig. 6B).

Finally, co-expression of i(CTG)480 with *miR-10* (*Mhc-Gal4 UAS-i(CTG)480>UAS-miR-10*) significantly increased lifespan compared with controls that co-expressed i(CTG)480 and GFP, resulting in an MS of 18 days (Fig. 6C and D; $P < 0.0001$). In addition, over-expression of *miR-10* alone (*Mhc-Gal4>UAS-miR-10*) did not affect the lifespan of flies (MS of 35 days; Fig. 6C).

Taken together, although further studies are required to fully understand the implications of *miR-1*, *miR-7* and *miR-10* dysregulation in CTG-mediated toxicity, our results demonstrate that *miR-10* down-regulation triggered by CTG expansions has a negative, but partially reversible,

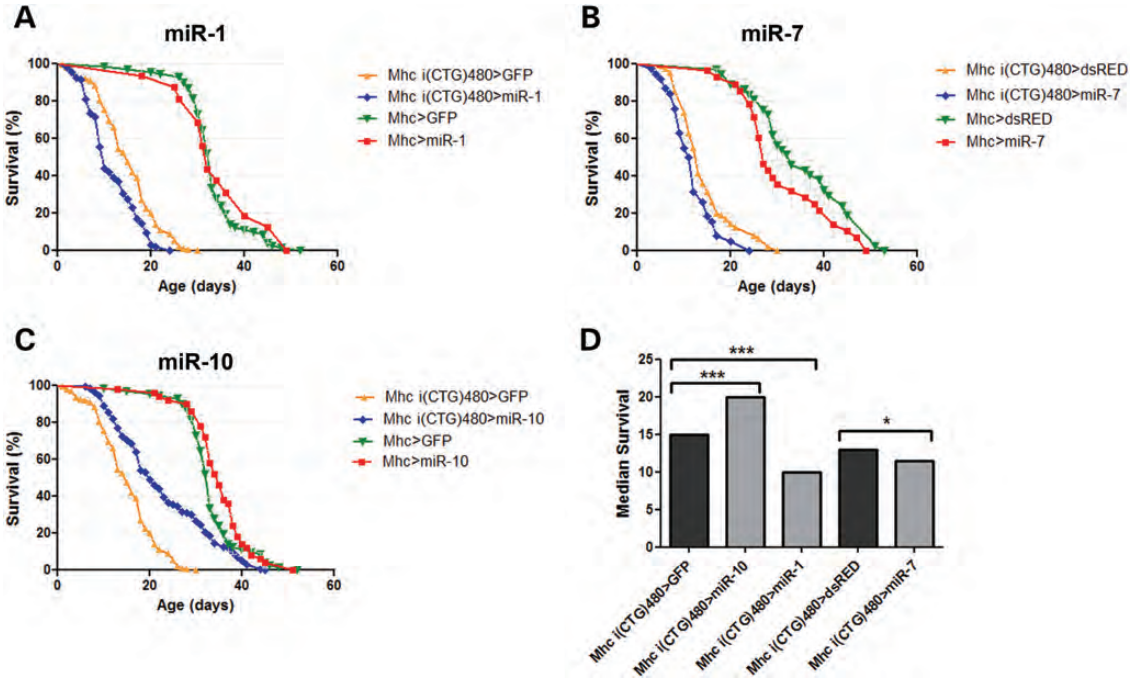

Figure 6. Overexpression of *miR-1*, *miR-7* or *miR-10* has different effects on the lifespan of CTG-expressing flies. Survival curves of flies co-expressing i(CTG)480 and *miR-1* ($n = 100$; **A**), *miR-7* ($n = 38$; **B**) or *miR-10* ($n = 180$; **C**), compared with control flies that co-express i(CTG)480 and GFP ($n = 161$; A and C) or i(CTG)480 and dsRED ($n = 89$; B). A mild but significant survival reduction was observed upon co-expression of i(CTG)480 and *miR-1* (**A**) or *miR-7* (**B**), whereas *miR-10* significantly extended the lifespan of CTG-expressing flies (**C**). Over-expression of *miR-1* ($n = 32$; A) or *miR-10* alone ($n = 35$; C) was not toxic compared with GFP ($n = 33$), whereas over-expression of *miR-7* alone reduced survival ($n = 27$; B) compared with dsRED ($n = 33$). (**D**) The graph showing the MS values obtained in (A)–(C). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

physiological impact, supporting the pathologic role and therapeutic potential of this miRNA in DM1.

DISCUSSION

Dysregulation of specific miRNAs in DM1 patients had been previously described (21,22,30). Here, we have studied the contribution of CTG expansions to miRNA defects in DM1, by analyzing changes in the muscle miRNome of a *Drosophila* model of CTG toxicity (2). Using SOLiD^{TM} 3 sequencing, we have identified 20 miRNA alterations caused by expression of CTG repeats. Of these, 19 were specifically down-regulated in our *Drosophila* model, whereas only 1 was up-regulated. Therefore, the alterations on miRNA regulation caused by CTG expression seem to trigger a reduction, rather than an increase, of miRNA expression levels. This effect was also observed in DM1 patients for all altered miRNAs that were conserved between *Drosophila* and humans: *miR-1*, an miRNA previously associated with DM1 (21,30); and *miR-7* and *miR-10a*, for which no previous link had been described.

Importantly, the conservation of *miR-1*, *miR-7* and *miR-10* defects between our fly model and DM1 patients confirms that: (i) the miRNA down-regulation found in *Drosophila* is specific, and not the consequence of a reduced contribution of the muscle transcriptome to the total transcriptome; and (ii) the dysregulation of these three miRNAs occurs in the presence of CUG-repeat transcripts devoid of additional *DMPK* sequences. Although it is possible that other coding or non-coding regions within the *DMPK* gene contribute to miRNA defects in DM1, this is the first demonstration that CTG expansions are directly linked to alterations in miRNA regulation. Of note, the fly model used in this work contains 480 CTG repeats interrupted every 20 units by the CTCGA sequence: i(CTG)480. The i(CUG)480 RNA is predicted to form a double-stranded structure that closely resembles the hairpin formed by 480 pure repeats, both of them having similar folding energies (Supplementary Material, Table S7). The existence of complex repeat interruptions at the DM1 locus has been reported to attenuate the severity of symptoms in patients (31–33). Although the CTCGA interruption in the i(CTG)480 transgene does not resemble any of these variant repeat alleles, it is possible that its presence might also modify CTG-induced phenotypes in our flies. For example, the CUCGA interruption would determine the length of any putative repeat-associated non-ATG (RAN) translation products, should these be generated in *Drosophila*, as i(CAG)480 transcripts would produce polyS, polyA and polyQ peptides in consecutive tracts of 20 amino acids linked by 1–2 amino acids. Note that RAN translation from pure CAG repeats produces individual polyS, polyA and polyQ peptides [Supplementary Material, Table S7 and (17,34)]. Bearing all this in mind, the conservation of *miR-1*, *miR-7* and *miR-10* defects between our fly model and DM1 patients represents important evidence that dysregulation of at least these three miRNAs occurs independently of the CUCGA repeat interruption in the *UAS-i(CTG)480* transgene.

By studying the expression levels of the predicted target genes of *miR-1*, *miR-7* and *miR-10* in skeletal muscles from DM1 patients, we identified a total of 42 targets that were dysregulated, 41 of them being up-regulated and only 1 down-regulated. The up-regulation of these targets is consistent with a reduced degradation by their respective miRNA regulators. qRT–PCR analysis confirmed this general trend, and validated at least seven of these alterations in DM1 patients, which had not been previously described to be triggered by miRNA dysregulation. Affected genes did not fall into related functional categories, but instead involved multiple cellular processes. Moreover, *miR-1*, *miR-7* and *miR-10* down-regulation could have an even higher impact on gene expression, if we take into consideration that these miRNAs might also affect the translation of additional gene targets, without affecting the levels of their messenger transcripts. Therefore, our results highlight the wide number of cellular mechanisms potentially affected by CTG-mediated disruption of miRNA regulation.

A number of miRNAs found altered in DM1 to date are encoded in introns, thus suggesting a link between pre-mRNA splicing and miRNA processing. Given that splicing alterations are a hallmark of DM1, both defects could have a common origin. In our study, two *Drosophila* miRNAs affected by CTG expression, *miR-1003* and *miR-1006*, are miRtrons. The precursor intron of *miR-1006* was completely spliced out both in control and in DM1 flies, suggesting that *miR-1006*-reduced levels in CTG-expressing flies do not originate from defects in the splicing regulation of its host transcript, but would instead occur at a more downstream level. For *miR-1003*, we found that its precursor intron is spliced out at higher levels in DM1 flies than in control individuals. However, mature *miR-1003* levels are reduced in DM1 flies. Increased levels of spliced-out *miR-1003* precursor could arise from a response mechanism triggered by the cells to compensate for the reduced levels of mature *miR-1003*, whereas the mature miRNA reduction itself would occur at a downstream level. In this study, we have also found altered miRNAs that belong to the same cluster (i.e. single-transcription units containing several miRNAs regulated by an upstream promoter) (35). In *Drosophila*, the pri-miRNA levels of clusters *miR-310–313* and *miR-959–964* were reduced in CTG-expressing flies compared with controls. Additionally, the levels of pri-miRNA for *miR-7*, but not for *miR-1* or *miR-10*, were down-regulated in CTG-expressing flies and in skeletal muscle of DM1 patients. Therefore, our results demonstrate that pri-miRNA transcription/stability is involved in at least part of the miRNA defects described in this work, supporting the idea of different origins for miRNA dysregulation in DM1.

In our DM1 model flies, the CTG-mediated reduction of *miR-1* seemed to be dependent on Mbl, as over-expression of MblC in CTG-expressing flies rescued *miR-1* levels. Moreover, *mbl* silencing in a wild-type background caused a strong reduction of *miR-1*. These results are consistent with previous reports that described a direct implication of MBNL1 in the biogenesis of human *miR-1* (21). In that study, the authors reported that MBNL1 binds to a UGC motif located within the loop of the pre-miRNA, facilitating the Dicer processing that generates the mature *miR-1*. According to this model, MBNL1 sequestration by CUG repeats would lead to a reduction of *miR-1* levels in DM1, which the authors validated in cardiac muscle from DM1 patients (2.1-fold reduction) (21), and is consistent with our results in flies and DM1 muscle biopsies. However, other reports have described a different situation for *miR-1*. When Perbellini *et al*. (30) measured *miR-1* from biceps muscles of DM1 patients, a 1.9-fold up-regulation of this miRNA, together with an increase in eight of its predicted targets, was found. This difference may be explained by the different types of muscles analyzed and/or their use of controls with suspected neuromuscular disorders. Intriguingly, another recent study reported no changes in *miR-1* levels in the *vastus lateralis* muscle of DM1 patients (22). It is, therefore, possible that *miR-1* dysregulation is particularly sensitive to cellular contexts, which could include factors such as the number of CTG repeats or the age of the patients.

In our experiments, *mbl* silencing also reduced *miR-7* levels. However, this reduction was weaker than that observed for *miR-1*. Moreover, over-expression of MblC did not rescue the effect of CTG expansions on *miR-7* levels. In our transdifferentiation cell model, *miR-7* levels were reduced both before and after myogenesis, whereas *miR-1* and *miR-10* were only significantly affected after differentiation. In addition, pri-miRNA down-regulation occurred for *miR-7*, but not for *miR-1* or *miR-10.* These observations further suggest that *miR-7* alterations in DM1 occur via a different mechanism, although further studies will be required to clarify the specific factors involved in each case.

The different behavior of *miR-1*, *miR-7* and *miR-10* in the presence of CTG expansions might translate into different consequences to the homeostasis of the cells. The pathological relevance of miRNA dysregulation in DM1 is unclear, as alterations previously described in miRNA levels could correspond either to a response mechanism or to a pathogenic consequence. Here we have shown that partial restoration of *miR-10* levels by over-expression of this miRNA in the *Drosophila* muscles partially rescued the reduced lifespan phenotype of DM1 flies. This demonstrates that *miR-10* down-regulation contributes to CTG-mediated toxicity. On the other hand, not all miRNA alterations triggered by CTG expression seemed to have a phenotypic impact, as over-expression of *miR-1* or *miR-7* did not rescue the CTG-induced phenotype, and even reduced the survival of flies. For *miR-7*, this effect could originate from additive toxicity, as *miR-7* over-expression alone affected the lifespan of flies. However, the case of *miR-1* is more intriguing, since this miRNA was not toxic *per se*. Given that human MBNL1 has been described to bind to *miR-1* directly, it would be possible that the CTG-specific detrimental effect observed for *miR-1* over-expression resulted from a sequestration of *Drosophila* Mbl by excess of *miR-1*.

In summary, this study sheds light onto our understanding of the molecular mechanisms behind gene expression dysregulation in DM1 and CTG toxicity, providing a direct link between miRNA dysregulation and RNA toxicity in DM1, identifying a number of mechanisms and predicted target genes that are affected by CTG expansions and supporting the pathogenic potential of at least part of them.

MATERIALS AND METHODS

Drosophila stocks

yw and *UAS-GFP* strains were obtained from the Bloomington *Drosophila* Stock Center (Indiana University). *UAS-i(CTG)480* and *Mhc-Gal4* flies were described in Garcia-Lopez *et al*. (2). Briefly, *UAS-i(CTG)480* flies expressed 480 synthetic CTG repeats interrupted every 20 units by the CTCGA sequence (Supplementary Material, Table S7). *UAS-MblC* flies were described in Garcia-Casado *et al*. (36). *UAS-IR-mbl* flies will be described elsewhere (37). *UAS-miR-1* flies were a gift from Dr Sokol (Dartmouth Medical School, USA) (38). *UAS-miR-7* flies were a gift from Prof. Cohen (Institute of Molecular and Cell Biology, Singapore, Singapore) (39). *UAS-miR-10* flies were a gift from Dr Gehrke (Stanford University School of Medicine, USA) (40). Flies used for SOLiD^{TM} 3 sequencing were fed with commercial instant *Drosophila* food (Carolina Biological Supply Company) in order to avoid variations in home-made food. All crosses were carried out at 25°C.

Small RNA library generation and next-generation sequencing

Two biological replicates per genotype were used (control: *Mhc-Gal4/+*; DM1: *Mhc-Gal4>UAS-i(CTG)480*), each of which containing 50 *Drosophila* males of the same age (2-day-old flies; state of muscle degeneration ∼45%). Two different DM1 lines were used (*UAS-i(CTG)480 1.1* and *UAS-i(CTG)480 2.2*) to rule out any transgene-specific effect (Supplementary Material, Table S1). Total RNA was extracted from each group and the small RNA fraction was enriched using the miRVana kit (Ambion). Small RNA was run in 15% acrylamide:bisacrylamide 19:1 gels and the 15–30 nt fraction was sliced out and eluted with 1 M NaCl overnight at 4°C. Purification was carried out using the MEGAclear Kit (Ambion). The quality of purified small RNAs (50 ng) was analyzed by capillary electrophoresis (Agilent 2100 Bioanalyzer). Libraries for SOLiD^{TM} 3 sequencing were prepared following the manufacturer's protocol (Small RNA Expression Kit, Applied Biosystems), and two technical replicates were conducted for *UAS-i(CTG)480 1.1* and *UAS-i(CTG)480 2.2* as a control of reproducibility (Supplementary Material, Table S1). Briefly, small RNA samples (15 ng) were hybridized and ligated overnight with the adapter mix, reverse-transcribed and PCR-amplified (15 cycles). The primers used in this PCR included a unique six-nucleotide barcode for each sample. A single emulsion PCR reaction was used to couple the barcoded libraries to P1-coated beads as per the standard Applied Biosystems protocol. After emulsion PCR, template beads were enriched in a glycerol gradient and deposited onto the surface of glass slides for SOLiD sequencing. Sequencing was performed using 35 bp chemistry on a version 3.0 SOLiD machine (SOLiD^{TM} 3). Approximately 200 million of 35 nt reads were produced for all barcoded samples.

Bioinformatic analysis

From the SOLiD^{TM} 3 sequencing data, low-quality reads were first removed from the data set (at least QV $\geq$ 10 in the first 10 bases). Filtered reads were then mapped against the *Drosophila melanogaster* genome (version r5.23), using the software Small_RNA_Tool_v0.5.0 (http://solidsoftwaretools.com/gf/) and allowing up to two mismatches in the first 18 nt and up to three mismatches in the entire read. A custom pipeline was then used to select reads that mapped uniquely to a point of the *Drosophila* genome, which represented the usable sequence data. mirBase (version 13.0) and custom scripts were applied in order to identify known miRNAs. Contaminations by protein-coding genes or other ncRNAs (rRNAs, tRNAS, snoRNAs, etc.) were discarded from the final data set. miRNA counts were normalized per million of reads that mapped uniquely, in order to yield the relative transcript abundance in the original sample. Normalized miRNA counts were used to analyze significant changes in expression profiles between *Mhc-Gal4>i(CTG)480* and *Mhc-Gal4/+* flies, using the dCHIP Analysis Software. Non-agglomerative hierarchical clustering was carried out using UPGMA (unweighted pair group method with arithmetic mean) analysis as previously reported (41). Statistically significant down- and up-regulated miRNAs were obtained using an ANOVA test with false discovery rate (FDR) correction ($\alpha = 0.05$).

Northern blot

For each biological replicate, total RNA from 50 adult male flies was extracted using Tri-Reagent (Sigma). The quality of the RNA was analyzed by capillary electrophoresis. Ten micrograms of total RNA was fractionated on a denaturing 15% polyacrylamide gel (7 M urea), electrotransferred to a Hybond-N^+ membrane (Roche) and fixed by ultraviolet cross-linking (1200 mJ). Membranes were probed with DIG-labeled LNA probes (EXIQON) complementary to the mature miRNAs or with 5′-DIG-labeled DNA probes (snoRNA U6, loading control) (Supplementary Material, Table S8) at 52°C overnight in hybridization buffer (36 mM Na_2HPO_4, 14 mM NaH_2PO_4, 1 mM EDTA and 7% SDS, pH 7.2). Anti-DIG-AP Fab fragment (Roche) was incubated at 1:10 000 for 1 h at room temperature. CDP-Star chemiluminescent substrate for AP (Roche) was used for detection. Images were taken in an ImageQuant LAS4000 (GE Healthcare). Quantification of band intensity was carried out using the ImageJ software. Pairs of samples were compared using a two-tailed *t*-test ($\alpha = 0.05$), applying Welch's correction when necessary.

qRT–PCR from flies

For each biological replicate, total RNA from 50 adult flies was extracted using Trizol (Sigma). One microgram of RNA was digested with DNaseI (Invitrogen) and retrotranscribed with SuperScriptII (Invitrogen), using random hexanucleotides. For each biological replicate, qRT–PCR reactions from 10 ng of cDNA were carried out per triplicate using SYBR Green PCR Master Mix (Applied Biosystems; Supplementary Material, Table S8). *tubulin84B* was used as the

endogenous control. Thermal cycling was performed in an ABi 7000 sequence detection system (Applied Biosystems). Relative expression to the endogenous gene and the control group was obtained by the $2^{-\Delta\Delta C_t}$ method. Pairs of samples were compared using a two-tailed *t*-test ($\alpha = 0.05$), applying Welch's correction when necessary.

Drosophila lifespan analysis

A total of 100–150 newly hatched flies per genotype were collected, placed in tubes containing standard nutritive medium and kept at 25°C. The number of deceases was scored on a daily basis, and flies were transferred into fresh medium every 3–4 days. Survival curves were obtained using the Kaplan–Meier method, and statistical curve comparisons were carried out according to the log-rank (Mantel–Cox) test ($\alpha = 0.05$).

DM1 patients and skeletal muscle biopsies

All biopsy specimens were taken after informed consent was obtained. Muscle biopsies used for qRT–PCR studies were collected at the University Hospital Donostia (San Sebastian, Spain) and at the University Hospital La Fe (Valencia, Spain), using institutionally approved protocols by an ethical board. A detailed description on muscle type, sex, age and number of repeats is provided in Supplementary Material, Table S9. For the determination of the CTG repeat size, genomic DNA isolated from peripheral blood leukocytes (42) and Southern blots probed with ^{32}P-labeled cDNA25 or PCR amplification of CTG-repeat regions (DM101 and DM102 primers) (43–45) were performed. Muscle biopsies used for the analysis of HUMAN EXON 1.0 ST arrays and subsequent qRT–PCR validation of mRNA targets will be published elsewhere.

qRT–PCR from human samples

Human muscle biopsies were homogenized in a Tissuelyser II (Qiagen), using QIAzol (Qiagen). RNA was then purified with a QIAcube (Qiagen), and the small RNA fraction was enriched using the miRNeasy Mini Kit (Qiagen). For mature miRNA analysis, 40 ng of RNA from five DM1 patients and three controls were used as a template for cDNA synthesis with TaqMan microRNAs RT (Applied Biosystems), following the manufacturer's recommendations. Twelve nanograms of the cDNA template was then amplified per triplicate by qRT–PCR, using specific stem–loop RT-type primers and TaqMan miRNA probes (Applied Biosystems; Supplementary Material, Table S8). qRT–PCRs assays were carried out in a 7900 HT Fast Thermal Cycler (Applied Biosystems) using the SDS software (version 2.2.2.). sno-RNA RNU48 was used as the endogenous control. For the analysis of human pri-miRNA and mRNA target levels, 1 μg of RNA from six DM1 patients and six healthy controls was used as a template for cDNA synthesis (RETROscript® Kit; Applied Biosystems). An amount of 20 ng of the cDNA template was then amplified per triplicate by qRT–PCR using SYBR Green PCR Master Mix (Applied Biosystems; Supplementary Material, Table S8). In this case, *GADPH* was used as the endogenous control. In all cases, relative expression to the endogenous gene and the control group was obtained by the $2^{-\Delta\Delta C_t}$ method. Pairs of samples were compared using a two-tailed *t*-test ($\alpha = 0.05$), applying Welch's correction when necessary.

Cell culture and qRT–PCR from transdifferentiated cells

Human fibroblasts were isolated from skin biopsies of a DM1 patient with 333 CTG repeats at the time of diagnosis (2010) and healthy individuals (Supplementary Material, Materials and Methods), after informed consent was obtained, according to the guidelines of the Committee on the Use of Human Subjects in Research of the Donostia Hospital (San Sebastian, Spain). Fibroblasts were cultivated in DMEM (Invitrogen), 10% FBS (Invitrogen) and antibiotics, in humidified incubators at 37°C in 5% CO_2. Transdifferentiation into myoblast-like cells was induced by turning on the myogenic program, using retroviral-mediated expression of murine MyoD under the control of the Tet-on inducible construct (42). Transduction experiments using lentiviral vectors were performed overnight in the presence of polybrene (4 mg/ml; Sigma-Aldrich), and transduction efficiency was confirmed by MyoD imunostaining 1 day after inducing differentiation. More than 80% of the cells expressed MyoD, using a multiplicity of infection (MOI) of 20 without vector-associated cytotoxicity (not shown). The lentiviral vector titer was determined by qRT–PCR. To induce differentiation, cells in confluence were transferred to DMEM supplemented with 2% horse serum, 1% penicillin/streptomycin, transferrin (100 μg/ml) and insulin (10 μg/ml; Sigma-Aldrich, St Louis, MO, USA), and doxycyclin (2 μg/ml; Sigma-Aldrich) was added in the differentiation medium. Before (day 0) and after (day 10) transdifferentiation was induced, cells were collected, and RNA extracted with the miRNeasy Mini Kit (Qiagen). qRT–PCR was performed using specific stem–loop RT-type primers and TaqMan miRNA probes as described above and in Supplementary Material, Table S8. Relative expression to the endogenous gene and to the control group was obtained by the $2^{-\Delta\Delta C_t}$ method. Pairs of samples were compared using a two-tailed *t*-test ($\alpha = 0.05$), applying Welch's correction when necessary.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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