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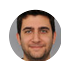

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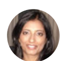

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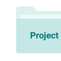

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Epigenetic changes as a common trigger of muscle weakness in congenital myopathies

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ABSTRACT

Congenital myopathies are genetically and clinically heterogeneous conditions causing severe muscle weakness and mutations in the ryanodine receptor gene (*RYR1*) represent the most frequent cause of these conditions. A common feature of diseases caused by recessive *RYR1* mutations is a decrease of ryanodine receptor 1 protein content in muscle. The aim of the present investigation was to gain mechanistic insight into the causes of this reduced ryanodine receptor 1. We found that muscle biopsies of patients with recessive *RYR1* mutations exhibit decreased expression of muscle specific microRNAs, increased DNA methylation and increased expression of class II histone deacetylases. Transgenic mouse muscle fibres over-expressing HDAC-4/HDAC-5 exhibited decreased expression of *RYR1* and of muscle specific miRNAs, while acute knock-down of *RYR1* in mouse muscle fibers by siRNA caused up-regulation of HDAC-4/HDAC-5. Intriguingly, increased class II HDAC expression and decreased ryanodine receptor protein and miRNAs expression were also observed in muscles of patients with nemaline myopathy, another congenital neuromuscular disorder. Our results indicate that a common pathophysiological pathway caused by epigenetic changes is activated in some forms of congenital neuromuscular disorders.

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INTRODUCTION

Congenital myopathies constitute a genetically and phenotypically broad spectrum of disorders characterized clinically by muscle weakness and atrophy, joint contractures, spinal deformities and variable cardiorespiratory involvement. Congenital myopathies have been historically defined by their most predominant histopathological feature, with major entities being Central Core Disease (CCD), Multi-minicore Disease (MmD), Nemaline Myopathy (NM) and Congenital Fibre Type Disproportion (CFTD) (1-4). Their severe complications require patients to receive continual medical attention, resulting in a substantial individual, familial and social disease burden. Each congenital myopathy can be caused by mutations in more than one gene and mutations in the same gene can cause different pathological phenotypes. The prime examples are *RYR1* -related myopathies, caused by mutations in the gene encoding the RyR1, the calcium release channel of the skeletal muscle sarcoplasmic reticulum. Physiologically, activation of the RyR1 leads to release of calcium from the sarcoplasmic reticulum, leading to muscle contraction by a process called excitation-contraction coupling (5). Excitation-contraction coupling occurs at the triad, a structure made up of two membrane compartments: the transverse tubules containing the voltage-gated dihydropyridine receptors and the sarcoplasmic reticulum terminal cisternae containing the ryanodine receptor Ca^{2+} release channels (RyRs). Excitation-contraction coupling requires the proper distribution and assembly of sarcoplasmic reticulum proteins and tight regulation of calcium homeostasis is critical for proper muscle function. Indeed, mutations in *RYR1* lead to calcium dysregulation and are the underlying cause of several neuromuscular disorders. While most dominant mutations associated with CCD and Malignant Hyperthermia

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Susceptibility are missense (6), recessive mutations associated with the pathological phenotypes of MmD, CNM and CFTD (1-4) are often compound heterozygous, with one allele presenting a non-sense, intronic splice site or a frameshift mutation, and the other allele presenting a missense mutation (3,4). As to their mode of action, dominant missense mutations affect the biophysical properties of the RyR Ca^{2+} channel (6), while for recessive mutations the mechanism is still elusive, though a common finding is the low levels of RyR1 and of other SR proteins in biopsied muscles (2-4, 7). Intriguingly, this decrease occurs only in mature muscle and not in other tissues expressing RyR1 such as B-lymphocytes (8).

Because of their heterogeneity, one of the major aims of research in congenital myopathies is to find a common target in order to develop a pharmacological tool to help improve muscle function and thus quality of life in this group of patients. In fact, though the number of patients with a given genetic form of a disease is small (1:3’000), the number of patients suffering from inheritable congenital myopathies worldwide is about 286 million (9) with central core disease accounting for 16% of cases, nemaline rod myopathy for 20%, centronuclear myopathy for 14% and multicore myopathy for 10% (http://www.muscular-dystrophy.org/research/patient_registries) (1). Thus discovering a common target downstream of the primary genetic defect could potentially benefit a large number of patients. The findings of the present investigation indicate that common epigenetic changes consequent to the primary genetic defect are activated in different congenital myopathies.

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RESULTS

Calcium homeostasis in myotubes from patients with mutations leading to decrease RyR1 content.

Our first approach was to study calcium homeostasis in myotubes derived from biopsies of four patients initially diagnosed as having Multiminicore disease, three of whom carry recessive *RYR1* mutations and one carrying the heterozygous mutation p.G297D but who exhibited reduced RyR1 protein on Western blot. Subsequent whole exome sequencing later revealed that the patient also harboured two compound heterozygous *NEB* mutations and is therefore identified as NEM UK06 in figure 1. Figure 1 shows that the resting fura-2 fluorescence ratio (340/380 nm) as well as the KCl-dependent calcium release curves were not different, except for patient Minicore UK 07, who showed a significantly reduced sensitivity to KCl (EC_{50} for KCl was 55.3±8.2 in myotubes from Minicore UK07 compared to 13.9±8.4 in myotubes from controls) (Figure 1A and B). The KCl-dependent calcium release curve for patient Minicore UK08 (*……* Fig. 1B) had also been previously reported to be similar to that of control myotubes (10). These results were surprising as Western blots of muscle biopsies from all four patients showed a very large reduction of RyR1 protein content (Figure 1 C) leading us to expect a large effect on calcium homeostasis in myotubes. Because of these reasons we hypothesized that the mechanism leading to reduced RyR1 expression is only operative in more mature tissues, such as myofibres, and not in cultured myotubes, even though the latter express the main protein components of the excitation -contraction coupling machinery (7).

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Epigenetic down-regulation of the ryanodine receptor 1

We focused the next series of experiments on epigenetic mechanisms that may be responsible for regulating RyR1 expression levels and in particular on the content of microRNAs (miRs). These endogenous ~22 nucleotide small non-coding RNAs are known to control gene expression by repressing translation or enhancing RNA degradation. Because of the limited amount of biological material available from patients, we decided to measure the expression levels of a selected group of muscle-specific miR transcripts, namely miR-1, miR-133, miR-206 and the muscle enriched miR-486 (11-13). miR-22, miR-124 were also measured as bioinformatics analysis showed that the 3'UTR of the *RYR1* gene contains binding sites for these two miRs As controls, we selected miR-126 and miR-221 that are reported to be expressed in many different tissues (14, 15). We analysed muscle biopsies from 5-9 controls, from 4-5 patients with CCD with dominant *RYR1* mutations, and from 12-16 patients with mutations leading to a decrease of RyR1 protein expression with pathological features of either MmD or CNM. The latter patients are categorized as "Minicore" and in addition to muscle weakness, they show decreased RyR1 protein in their muscle biopsy, and all have the following common pathological features: several minicores and increased number of internal nuclei in their muscle biopsy as well as a myopathic face often accompanied by ophthalmoplegia (16). All but patients Minicore NL01, UK02, UK05 and UK09 harboured recessive *RYR1* mutations (see Supplementary Table 1 for patient's diagnosis, genotypic and phenotypic characteristics). Figure 2 shows that the relative contents of miR-22, miR-124, miR-1 and miR-133 were reduced to almost undetectable levels in biopsies from >60% of Minicore patients with decreased RyR1 protein expression, while the reduction was not as consistent in biopsies from CCD patients. Our results also indicate that the observed changes in

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miRs are specific to some muscle transcripts and not caused by a more global defect in the miR synthesizing machinery as miR-206, miR486 as well as miR-126, and miR-221 were not decreased (Figure 2 and Supplementary Figure 1).

***RYR1* Methylation**

The results obtained so far point to a potential role of regulation of skeletal muscle gene expression by additional factors. We next verified whether changes of DNA methylation occur within the *RYR1* gene. This experiment is essential as methylation-dependent expression of the *RYR1* gene was not unexpected considering the presence of several CpG-rich regions. Genomic DNA was extracted from biopsies of four controls and four Minicore patients and the methylation of CpG regions was studied using the methyl-sensitive HpaII and the methyl-insensitive MspI restriction enzymes. After cleavage of genomic DNA, quantitative real-time PCR was performed and compared with a control PCR amplifying a proximal *RYR1* gene region lacking HpaII/MspI cleavage sites (see schematic representation in figure 3A). The results obtained clearly indicate that the CpG-III region of the *RYR1* gene (from nucleotide 6790 to nucleotide 7035) of all the Minicore patients analyzed is hypermethylated compared to that of controls (Figure 3B) suggesting that *RYR1* mutations are associated with deep changes in the pattern of DNA methylation. Additionally, we analysed biopsies from Minicore patients and controls for DNA methyltransferase expression and found that in the former group DNMT1 and DNMT2 are significantly up-regulated (Figure 3C), whereas expression of DNMT3 did not vary significantly between controls and patients (results not shown).

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HDAC expression levels in muscle biopsies of patients with Minicores with recessive *RYR1* mutations

The levels of expression of HDAC-4 and HDAC-5 in muscle biopsies from controls and patients were subsequently determined for the following reasons: (i) class II histone deacetylases (HDACs) can be recruited in association with DNA-methylation; (ii) these enzymes repress transcription by deacetylating core histones (17); (iii) they affect myogenesis by binding to the muscle transcription factor mef2 (18); (iv) they are predominantly expressed in those tissues expressing mef2, that is skeletal muscle, heart and brain (18) and (v) HDAC-4 is a target of miR-22 whose down-regulation potentiates HDAC-4 expression (19). Figure 4A shows a representative Western blot of total muscle homogenate stained with anti-HDAC-4 and HDAC-5 antibodies; the bottom lane shows a loading control of the same blot stripped and probed with anti-myosin heavy chain antibodies (MHC) recognizing all MHC isoforms. The control biopsy shows low levels of class II HDACs (lane 1 Figure 4A), whereas samples from the Minicore patients contain abnormally high levels of HDAC-4 and HDAC-5. Figure 4B shows the relative content of HDAC-4 and HDAC-5 normalized for MHC content in biopsies from all the available patients. Biopsies from patients with recessive compound heterozygous *RYR1* mutations, with a non-sense, intronic splice site or a frameshift mutation in one allele and a missense mutation in the other allele show a six- to 15-fold increase in class II HDAC content and the increase in HDAC-4 expression was also detectable at the transcriptional level (Figure 4D). Interestingly, HDAC-4 was not detectable in any of the myotube cultures (Figure 4C) supporting the observed lack of effect of the mutations on the excitation-contraction coupling characteristics of myotubes from the Minicore patients (Figure 1). Figure 4E shows a photomicrograph taken by confocal microscopy on a sample

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from Minicore SA06; as can be seen, though the vast majority of HDAC-4 is distributed throughout the muscle fibre, the amount co-localizing with nuclei is higher in the patient's biopsy than in the control biopsy. Analysis of two Minicore patients and two controls confirmed that the percentage of HDAC-4 co-localizing with nuclei is approximately 10 times higher in muscles from Minicore patients than from controls (9.2 ±3.8% vs 1.1±0.3%, respectively). We also compared HDAC-4 and HDAC-5 up-regulation and *RYR1* hypermethylation in the samples of the four Minicore patients and found a positive correlation between high HDAC-4/5 levels and hypermethylation of the studied *RYR1* CpG-III gene sequence (Figure 5A and B).

Effect of HDAC4/5 over-expression and *RYR1* silencing in mouse muscle fibres.

In order to demonstrate a causative link between *RYR1* mutations and the above described epigenetic changes, we manipulated gene expression by creating transgenic intact adult mouse skeletal muscle *flexor digitorum brevis* (FDB) fibres by either (i) over-expressing HDAC-4 and HDAC-5 or (ii) knocking down RyR1 by siRNA silencing. When compared to acute transfection with an empty plasmid, acute over-expression of HDAC-4 and HDAC-5 directly recapitulates the effects observed in muscle biopsies from Minicore patients (Figure 6). That is, acute over-expression of HDAC-4 and HDAC-5 in mouse FDB fibres decreases *RYR1* transcript expression by approximately seventy percent (figure 6) and RyR1 protein content by 75% (Supplementary Figure 3), down-regulates muscle-specific miRs and down-regulates the expression of myomesin-1, a muscle specific gene whose expression is regulated by the transcription factor mef2 (20, 21)(Figure 6). No changes were observed in mef2, miR-126, miR-221 and miR-486 expression (Supplementary table 2). On the

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other hand, silencing *RYR1* for eight days with siRYR1 significantly increased HDAC-4 and HDAC-5 expression levels but did not change the expression of muscle-specific miRs (Figure 7).

Increasing class II HDAC expression does not affect mef2 expression

Having established that down-regulation of *RYR1* causes an increase in HDAC-4/5 expression, we reasoned that this elevation could lead to downstream effects on mef2, a master trans-activator of skeletal muscle gene expression (22). Mef2 is sequestered by class II HDACs resulting in blockage of mef2 dependent gene transcription (23) and the *RYR1* and miR1/miR-133 genes contain intragenic mef2-dependent enhancer sequences that regulate their transcription in muscle (24, 25). We therefore examined the patient's muscle biopsies for: (i) mef2 content, to ascertain that there was no compensatory up-regulation of its expression due to the increased expression of HDAC-4/5 and (ii) myomesin a protein that is transcriptionally regulated by mef2 (20, 21). Figure 8 shows that the transcript levels of MEF2A, MEF2C and MEF2D are not significantly different between controls and biopsies from patients. On the other hand, myomesin-1 protein levels normalized to MHC are significantly decreased (by approximately fifty per cent in biopsies isolated from patients with Minicores (Figure 8B and C).

HDAC4/5 are also up-regulated in other congenital myopathies

In order to verify whether these observed effects (that is increased levels of HDACs, decreased levels of RyR1 and decreased levels of muscle specific miRs) are specific for congenital myopathies due to recessive *RYR1* mutations or a more general response occurring in patients with other congenital myopathies, we analysed biopsies

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from 11 patients with nemaline myopathy harbouring mutations in *KBTBD13*, *ACTA1* or *NEB* (NEM 6, NEM3 and NEM2, respectively). A similar decrease in muscle-specific miR-22, miR-133 and miR-1s was observed (Supplementary Figure 2). We also tested the muscle biopsies from the patients with nemaline myopathy for RyR1 content and HDAC-4 and HDAC-5 expression. Surprisingly, RyR1 protein content was significantly reduced (control vs. NEM was 100± 27.9% vs 0.03± 0.02%, $p<0.01$, Student's *t* test) and HDAC-4 and HDAC-5 protein levels were significantly increased (Supplementary Figure 2).

DISCUSSION

Here we identify a novel pathophysiological mechanism occurring in skeletal muscles of patients with congenital myopathies whereby activation of a cascade of events leads to the down-regulation of muscle specific genes. We report that recessive compound heterozygous *RYR1* mutations are accompanied by the following changes in skeletal muscle: (i) hypermethylation of the *RYR1* gene, (ii) a six to fifteen fold increase in class II HDAC expression and (iii) reduction in muscle-specific miRs. Our results represent a major advancement in the field as to date the mode of action of recessive *RYR1* mutations identified in patients with MmD, CNM and CFTD has been elusive and the functional characterization of cells harbouring such mutations has failed to yield a mechanism compatible with the disease phenotype (8, 10, 26). A regular finding in muscle biopsies of patients with recessive *RYR1* mutations has been a reduced expression level of RyR1 protein and transcript (2-4, 26-28). This reduction appears to be muscle- specific and has not been observed in other tissues ectopically expressing RYR1, such as B-lymphocytes (8). Because of these results, we set out to

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test the hypothesis whereby the mechanism leading to reduced RyR1 muscle expression may be epigenetically regulated and the schematic representation depicted in Figure 9 summarizes the results of the present study. The key point is that *RYR1* mutations are accompanied by an increased expression of class II HDACs. We are aware that an increase in HDAC expression has been reported in other conditions, including denervation, muscle atrophy, ALS and Huntington's disease (29-34). However, we would like to point out that (i) such high levels of expression of class II HDACs have not been reported in any other human neuromuscular abnormalities investigated so far; (ii) such an increase of HDAC is specific since the signal to background ratio of our set of data is 6 to 16 times greater then reported in other muscle disorders and (iii) over-expression of HDAC-4 and HDAC-5 in mouse FDB fibers results in the down-regulation of RYR1 and of muscle-specific miRs. High levels of HDACs not only lead to chromatin condensation thereby decreasing gene transcription (21, 35, 36), but also sequester mef2 (23, 37-39). In this context it should be pointed out that there is no compensatory up-regulation of mef2 in muscles from the patients, so that up-regulation of HDACs would lead to the down-regulation of mef2 dependent proteins. That this is the case is supported by the fact that *RYR1*, myomesin and muscle-specific miRs containing mef2 dependent binding domains (21, 24, 25) are significantly down regulated in the muscles of Minicore patients. Though few studies have focused on the mechanisms regulating RYR1 expression, the 5' region of the human and porcine *RYR1* gene contains, aside a mef2 binding domain (24), consensus sequences for the transcription factor SP1, for muscle specific promoter elements as well as for a number of transcriptional activators (40). SP1 is a zinc finger transcription factor that binds to CG rich regions present in many promoters. In fibroblasts SP1 interacts with HDAC2 leading to the transcriptional

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silencing of the human telomerase reverse transcriptase (hTERT) gene in normal somatic cells (41). Whether SP1 can also interact with class II HDACs and whether this interaction is modified by CpG methylation also leading to repression of *RYR1* gene transcription remains to be investigated.

Interestingly the 3'UTR of HDAC-4 and of HDAC-5 have binding sites for miR-22/mir-124/miR-1/miR-206 and miR-206, respectively and HDAC-4 is a target of miR-22 whose down-regulation potentiates its expression (19). Thus it follows that a decrease of miR-22, miR-124 and miR-1 activates a pathological loop leading to the further up-regulation of class II HDACs (Figure 9). This mechanism is compatible with and gives mechanistic insight to two previous observations: (i) other muscle specific genes besides the *RYR1* are down-regulated in patients with congenital myopathies due to *RYR1* mutations (7); (ii) the *RYR1* was reported to be imprinted in some patients with MmD because of epigenetic factors (42). The former observation is likely due to the sequestration of mef2 by class II HDACs (37-39). The latter observations on the other hand can be explained by the finding that the DNA methyltransferases DNMT1 and DNMT2 are over-expressed in muscle biopsies of Minicore patients, bringing about *RYR1* hypermethylation. DNMT1 is a maintenance methyltransferases preserving methylation patterns but also has *de novo* activity (43). DNMT2 on the other hand is thought to participate in the recognition of damaged DNA and mutation repair (44). In fact, the two observations are mechanistically linked as hypermethylation goes hand in hand with HDAC activation and gene down-regulation (23, 24, 35, 36, 45); furthermore DNA damage can activate DNA methylation via activation of DNMT1 (46), resulting in a pathological loop that will ultimately shuts down gene transcription of mef2-dependent genes.

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As to the role miRs in neuromuscular diseases, this is still unclear: over-expression of miR-22 is sufficient to cause cardiomyocyte hypertrophy (47) and several miRs are up-regulated in muscular dystrophies (48-50) but depending on the disease some miRs may appear to be down-regulated (50). Interestingly, a recent study demonstrated that mice lacking miR-133 develop an adult onset centronuclear myopathy in type-2 fibres and this is accompanied by impaired mitochondrial function, fast to slow myofiber conversion and disarrangement of triads (51), histopathological changes very similar to those observed in recessive human *RYR1*-related myopathies. Though in the paper the authors conclude that this is principally due to the dysregulation of dynamin-2, one of miR-133's targets, the similarities between the phenotype of the miR-133a knock out mice and that of patients with *RYR1* mutations is striking and is indicative of a common pathophysiological pathway.

The main point emerging from our studies is that epigenetic factors are central culprits in recessive *RYR1*–linked myopathies. Our results also show that these factors are likely to also play a major role in other congenital muscle diseases such as nemaline myopathy. In support of a role of epigenetics, moderate exercise has been shown to improve the muscle function of some patients with congenital myopathies (52, 53) and there is increasing evidence that physical activity influences DNA methylation in humans (53, 54). Taken together our results suggest that a common pathophysiological mechanism is activated in skeletal muscles of patients with some congenital myopathies. The presence of mutations in muscle specific genes (in this case *RYR1*) activates factors that lead to the up-regulation of DNA methyltransferases and of class II HDACs, the master regulator of chromatin structure. Though the primary mechanism causing HDAC up-regulation is at the moment unclear, our data

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provide the proof of concept that DNA methyltransferase and HDAC are potential pharmacological targets to treat a wide range of inherited neuromuscular conditions with different genetic backgrounds that as a common feature lead to a decrease in RyR1.

MATERIALS AND METHODS

Quantitative PCR: Total RNA was extracted using Trizol (Life Technologies, #15596018). cDNA was synthesized with the High Capacity cDNA synthesis kit or Taqman microRNA Reverse Transcription kit (Applied Biosystems, #4366596). Transcript levels were quantified using Syber-Green reagent on an Applied Biosystem platform (7500 fast real-time PCR system); levels of expression from triplicate replicas were averaged and normalized to the content of the muscle-specific gene desmin (*DES*). In the case of human samples, because of the limited amount of biological material, not all biopsies could be investigated for all genes. The sequences of the primers used for qPCR are listed in Supplementary Table 3.

MicroRNA determination: Quantification of selected miRs was performed using TaqMan master mix no-UNG 2 (Life Technologies, # PN 4427788) and the following miR assays (Life Technologies, # PN 4427975): miR-22, miR124a, miR-133a, miR-1, miR-206, miR-486-3p, miR-221 and miR-126. Each reaction was performed in triplicate and the results from each muscle sample were analysed and averaged. In human muscle biopsies, miR expression levels were normalized to RNU44 and to the muscle specific genes Desmin (*DES*) and Actinin2 (*ACTN2*) that show similar Ct values in patients and healthy individuals. In mouse FDBs miR expression levels were normalized to U6 snRNA. In the case of human samples, because of the limited

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amount of biological material, not all biopsies could be investigated for all microRNAs.

DNA methylation: Total genomic DNA was isolated using the GeneElute mammalian genomic DNA Miniprep kit (Sigma Genosys). DNA methylation was assessed by PCR amplification of genomic DNA digested with the methyl-sensitive HpaII and MspI restriction enzymes (55)(see schematic representation in Fig.3A). Restriction enzyme digestion reactions were carried out overnight at 37°C, with HpaII or MspI (New England BioLabs), in twenty µl final volume. The primers used for PCR amplification are listed in Supplementary Table 3 as: Human RYR1 CG-rich (F and R) amplifying the CpG-III region of the *RYR1* gene and Human RYR1 Control (F and R), amplifying a control region of the *RYR1* gene lacking HpaII/MspI sites (see Fig. 3A). To determine the extent of DNA methylation, the ΔCt values were first obtained comparing the CpG-III and control PCRs of HpaII digestions, then the ΔΔCt values were generated using as reference the samples exhibiting the higher ΔCt. Higher ΔΔCt values indicate higher extent of DNA methylation at the CpG-III RyR1 MspI/HpaII cleavage sites.

***Electrophoresis and Immunoblotting*:** Total muscle proteins were extracted in 10 mM Hepes pH 7.0, 150 mM NaCl, 1mM EDTA and anti-protease (Roche, # 11873580001). Protein concentration was determined using Protein Assay Kit II (Bio-Rad Laboratories) using BSA as a standard. SDS PAGE, protein transfer on to nitrocellulose membranes and immunostaining were performed as described previously (2, 8). The following primary antibodies were used: mouse anti-RyR1 (Ryanodine 1 Receptor, Thermo Scientific, # MA3-925), mouse anti-MHC (myosin heavy chain, Millipore, #05-716), Rabbit anti- HDAC-4 (Histone Deacetylase 4, Cell

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Signaling, #2072), rabbit anti- HDAC-5 (Histone Deacetylase 5, Abcam #1439), Rat anti-myomesin was a generous gift of Prof. Mathias Gautel, King's College, London, UK (56). Secondary peroxidase conjugates were Protein G–peroxidase (Sigma, #P8170) and peroxidase-conjugated goat anti-mouse IgG (Sigma, #A2304). The immunopositive bands were visualized by chemiluminescence using the Super Signal West Dura kit (Thermo Scientific). In order to perform statistical analysis, the intensity of the immunopositive bands were determined using ImageJ/FIJI. The intensity values were normalized to the intensity of the indicated muscle-specific house-keeping protein. The value (arbitrary units) obtained from the patient's biopsies were divided by the mean value obtained from control biopsies and are expressed as 100%.

***Mouse muscle fibre electroporation and isolation*:** The procedure was as described by DiFranco *et al*. (57). Briefly, eight-14 week old mice were anesthetized using isofluorane and 7.5 µl of 2mg/ml Hyaluronidase in RNase free Tyroide's Buffer (Sigma Fine Chemicals, #H3506) was injected under the footpad. The mice were left one hour under supervision and subsequently the following constructs were injected into the footpad: pCMV6-HDAC4 (Origene #MR211598), pCMV6-HDAC5 (Origene # MC202550) while control mice (mock transfected) received twenty µg of pIRES2-dsRed2 plasmid (Clonetech # 632420). For siRNA silencing experiments 6 nmol of RNA either specific for the RYR1 (Ambion; siRNA RyR1-#4390771), or a scrambled siRNA sequence (Negative control 2- #4390845) were used. siRNA transfected FDBs were also injected with lipofectamine RNAiMAX (Invitrogen, #13778-030). Ten minutes post injection, FDBs were electroporated using acupuncture needles placed parallel and perpendicular to the long axis of the foot (with one cm distance) and twenty pulses (100v/cm, 20ms duration and 1Hz of frequency) were given. Six to ten

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days post transfection the mice were sacrificed and FDBs were isolated by enzymatic dissociation at 37°C for 60 min in Krebs Ringer solution no Ca^{2+} (pH 7.4), containing 0.2% collagenase I (Sigma Fine Chemicals, C-0130). Enzymatic digestion was terminated by washing the muscle with Tyrode's solution (pH 7.4) and single fibres were isolated and total protein extracts prepared or RNA was extracted and analysed by qPCR.

Ca^{2+} measurements**:** Primary skeletal muscle cultures and cell imaging were performed as previously described (58).

Confocal Microscopy and Immunofluorescence**:** Biopsies were embedded for pathological examination and sliced using a cryostat (ten µm thickness). Cryosections were fixed with methanol: acetone (1:1) for thirty minutes and then incubated in the following solutions for ninety minutes at room temperature: blocking solution (Roche, #115000694011), rabbit anti- HDAC-4 (Cell Signaling, #2072), Alexa Fluor 647 conjugated anti-Rabbit IgG (Life Technologies, #A21245). Nuclear staining was performed using DAPI (Invitrogen, #D21490) and slides were mounted with mounting medium (Sigma, #1000-4) and sealed hermetically with 1.5 mm thick coverslip. A Nikon A1R Confocal microscope was used for 3D image acquisition with a 40X oil objective (N.A = 1.3). Images were analysed using threshold co-localization function in ImageJ2/FIJI program.

Compliance with Ethical standards**:**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethikkommission beider Basel (permit N°

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EK64/12); all subjects gave written informed consent to carry out this work.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Experiments on mouse muscles were approved by the local Cantonal Veterinary authorities (permit N° 2658).

Statistical analysis and graphical software**:** Statistical analysis was performed using the Student's *t* test; means were considered statistically significant when the p value was <0.05. When more than two groups were compared, analysis was performed using the ANOVA test followed by the Bonferroni post-hoc test using the statistical package included in GraphPad Prism 6.0 software. Origin 6 was used to generate dose–response curves. Images were assembled using Adobe Photoshop CS (version 8.0).

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Conflict of interest: The Authors have declared that no conflict of interest exists.

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FIGURE LEGENDS

Figure 1: Myotubes from Minicore patients harboring recessive *RYR1* mutations do not show alterations of the resting [Ca^{2+}] nor decreased Ca^{2+} release after *in vitro* stimulation. A. Fura-2 loaded myotubes were imaged in Krebs Ringer solution containing 2 mM Ca^{2+}. No difference in the resting $[Ca^{2+}]_i$ was observed between controls (white bar), cells from patients with recessive mutations (grey bars) or a patient initially diagnosed as MmD carrying the heterozygous p.G297D *RYR1* mutation but who also carries 2 compound heterozygous *NEB* mutations (black bar). Bars represent the mean (± SEM) fluorescence (340/380 nm) from the indicated number of cells. **B.** KCl dependent peak Ca^{2+} release in Krebs Ringer containing 100 µM La^{3+} Each point represents the mean (±SEM) increase in fura-2 fluorescence ratio (340/380 nm) of at least 10 myotubes. The data was analyzed through Bolzmann equation using Origin 6.0. **C.** Western Blot analysis of total protein extracts from muscle biopsies shows major decrease in RyR1 protein expression in Minicore patients. Desmin served as loading control.

Figure 2: Muscle-specific miR expression levels differ in biopsies from patients with dominant and recessive *RYR1* mutations. Each symbol represents the mean relative expression of the indicated miR from a single patient normalized to RNU44 content and to the muscle specific housekeeping genes (*DES/ACTN2*). Control healthy individuals, circles; CCD with dominant *RYR1* mutations, squares; Minicore, triangles. Statistical analysis was performed using ANOVA and Bonferroni multiple comparison test (95% Confidence interval). **miR22** *$p<0.0005$, CTRL n=9, CCD

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n=4, Minicore n=15; **miR124** **p< 0.05, CTRL and CCD n=5, Minicore n=12; **miR1***p<0.0005, ***p< 0.009, CTRL n=8, CCD n=5, Minicore n=16; **miR133** ****p<0.002, CTRL n=10, CCD n=4, Minicore n=16; **miR206**; no statistical significance change between groups. CTRL n=9, CCD n=5, Minicore n= 16. Statistical analysis was performed using ANOVA and Bonferroni multiple comparison test (95% Confidence interval).

Figure 3: The *RYR1* is hypermethylated and DNA methyltransferases 1 and 2 are up-regulated in muscles from Minicore patients. A. Schematic representation showing the location of the CpG region III within the *RYR1* gene, the position of the CpG sites (indicated by arrowheads), and the location of the 5'-CCGG-3' HpaII/MspI site (arrowed); the location of the internal control region, lacking HpaII/MspI sites is also shown, as well as the location of the PCR primers used to amplify the DNA. **B.** Hypermethylation of CpG region III of the *RYR1*. Each symbol represents the mean relative methylation value from a patient (CTRL n=4; Minicore n=4). Experimental details are outlined in the methods section. **C.** DNA methyltransferase 1 (DNMT1) and DNMT2 are significantly up-regulated in muscles of Minicore patients. Each symbol represents the mean relative expression of DNMT1 (CTRL n=5; Minicore n=9) and DNMT2 (CTRL n=4, Minicore n=8) from a single patient normalized to the muscle specific housekeeping gene *DES (** P< 0.02, **P<0.025, Student's *t* test).

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Figure 4: **Class II HDACs are significantly up-regulated in muscle biopsies from patients with Congenital Muscle Disorders**. A.Western blot analysis of biopsies from a control muscle (lane 1) and Minicore patients (Minicore SA03 and Minicore SA05). Fifty micrograms of total muscle protein extracts were separated on a 6 % SDS PAG, blotted onto nitrocellulose and probed with anti-HDAC-4 and HDAC-5. Lower portion of the figure, loading control; the same blot was probed with anti-MHC recognizing all isoforms. **B.** Quantification of HDAC-4 and HDAC-5 normalized to MHC in muscle biopsies of controls (circles), CCD patients (squares) and Minicore patients (triangles). **HDAC4** *$p<0.001$, CTRL n=9, CCD n=5, Minicore n=12; **HDAC5****$p<0.01$ CTRL, n=4, CCD n=3, Minicore n=9; ANOVA and Bonferroni multiple comparison test were performed. Each symbol represents results from a single patient. **C.** No HDAC-4 protein is detectable in myotubes. **D.** HDAC4 transcript levels as assessed by qPCR in muscle biopsies from controls and Minicore patients ***$p<0.02$, CTRL n=4, Minicore n=14; statistical analysis performed using Student *t* test. **E.** Confocal microscopy showing distribution of HDAC-4 in a muscle biopsy from a control (left panel) and Minicore patient. Arrows indicate co-localization of HDAC-4 and DAPI. Bar indicates 10 μm.

Figure 5: Correlation between DNA methylation and HDAC-4/HDAC-5 expression and up-regulation of DNA methyltransferases in muscles of patients with Minicore. The data shown in this figures was obtained from biopsies from 4 control individuals (empty squares) and from biopsies from 4 Minicore patients (filled squares) (Minicore NL02, Minicore NL03; Minicore SA03, Minicore SA05). **A.**

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Correlation between HDAC-4 and *RYR1* hyper-methylation (correlation coefficient r =0.4695). **B**. Correlation between HDAC-5 and *RYR1* hyper-methylation (correlation coefficient r = 0.7925).

Figure 6: In vivo over-expression of HDAC-4 and HDAC-5 causes down-regulation of RYR1 and of muscle-specific miRs. Each symbol shows the mean triplicate relative expression value of the indicated transcript normalized to the indicated gene. Circles, control FDB fibers mock transfected with the empty pIRES2-dsRed2 plasmid; squares, FDB fibers transfected with a plasmid encoding mouse HDAC-4 and HDAC-5. **HDAC4**; *p<0.0001, n=6; **HDAC5** **p<0.005, n=6; **RYR1** **p<0.005, CTRL n=4, HDAC4 & 5 n=6; **miR22 and miR1**; ***p<0.045, n=6; **miR133** ****p<0.035, n=6; **miR206** ****p<0.035, n=5; **Myomesin** ****p<0.035, n=4; **miR124** was quantified and no significance change was observed. Statistical analysis was performed using the Student's *t* test.

Figure 7: Down-regulation of RYR1 by siRNA leads to up-regulation of HDAC-4 and HDAC-5. Each symbol shows the mean triplicate value relative expression level of the indicated transcript normalized to U6 snRNA (miR expression) or *DES* (*RYR* and *HDAC* expression), in fibers isolated from a single mouse. Circles, control (mock) transfection with a scrambled siRNA; squares FDB transfected with siRYR RNA (see Methods section for details). **RYR1** *p<0.0001, n=7; **HDAC4****p<0.04, CTRL n=6, siRYR1 n=7. **HDAC5**; **p<0.04, CTRL n=6, siRYR1 n=9. **miR22**, **miR133**, **miR124** and **miR1** show no statistical difference. Statistical analysis was performed using the Student's *t* test.

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Figure 8: Increased expression of HDAC-4 /HDAC5 leads to a decrease in the content of myomesin, without affecting mef2. A. mef2A, mef2C and mef2D transcript levels are similar in muscle biopsies of patients and controls (expression levels normalized to *DES*)**. B.** Representative Western blot showing that the protein content of myomesin is lower in patients with Minicore compared to healthy individuals (letters and numbers refer to patient N° see Supplementary Table 1); **C.** Quantification of myomesin protein content in muscle biopsies: controls, circles (n=6); CCD, squares (n=5); Minicore, triangles (n=9). *p <0.02. Statistical analysis was performed using ANOVA and Bonferroni multiple comparison test (95% Confidence interval).

Figure 9: Cartoon depicting how mutations in *RYR1* lead to a decrease in RyR1 content thereby leading to weak muscles. Mutations lead to DNA hyper-methylation and HDAC-4/HDAC-5 over-expression. This causes mef2 sequestration thereby inhibiting transcription of genes regulated by mef2, including the *RYR1* and muscle-specific miRs. A decrease in RyR1 would severely affect muscle excitation-contraction coupling since this calcium channel is a central player in this mechanism, releasing the calcium necessary for muscle contraction from the sarcoplasmic reticulum.

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ABBREVIATIONS: Central core disease, CCD; Centronuclear myopathy, CNM; Congenital Fibre Type Disproportion, CFTD; DNA methyltransferase, DNM; Excitation-contraction coupling, ECC; Histone de-acetylase, HDAC; *flexor digitorum brevis*, FDB; micro RNAs, miRs; Multiminicore disease, MmD; myosin heavy chain, MHC; Nemaline Myopathy, NM; Ryanodine receptor1, RyR 1.

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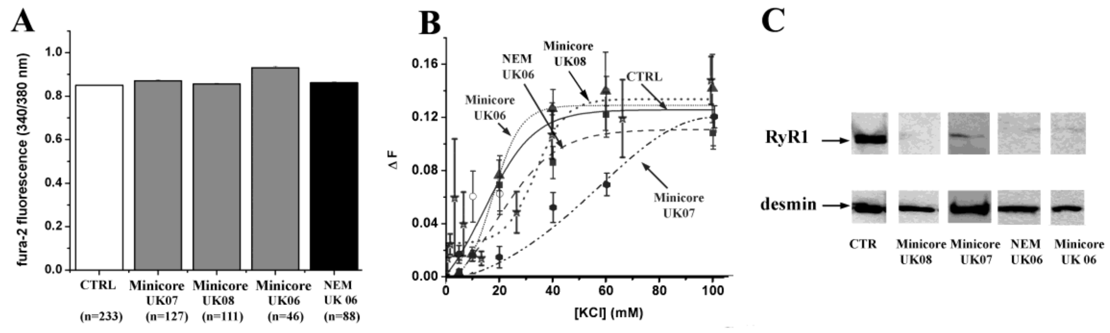

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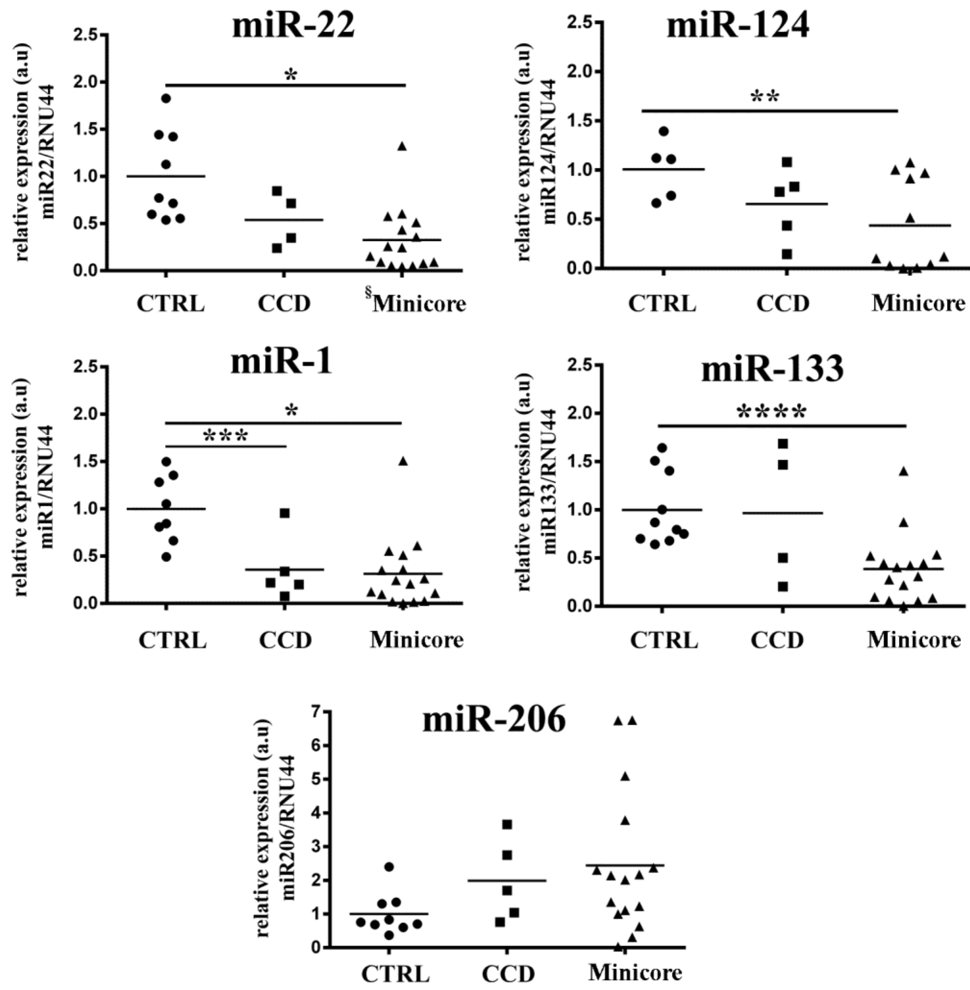

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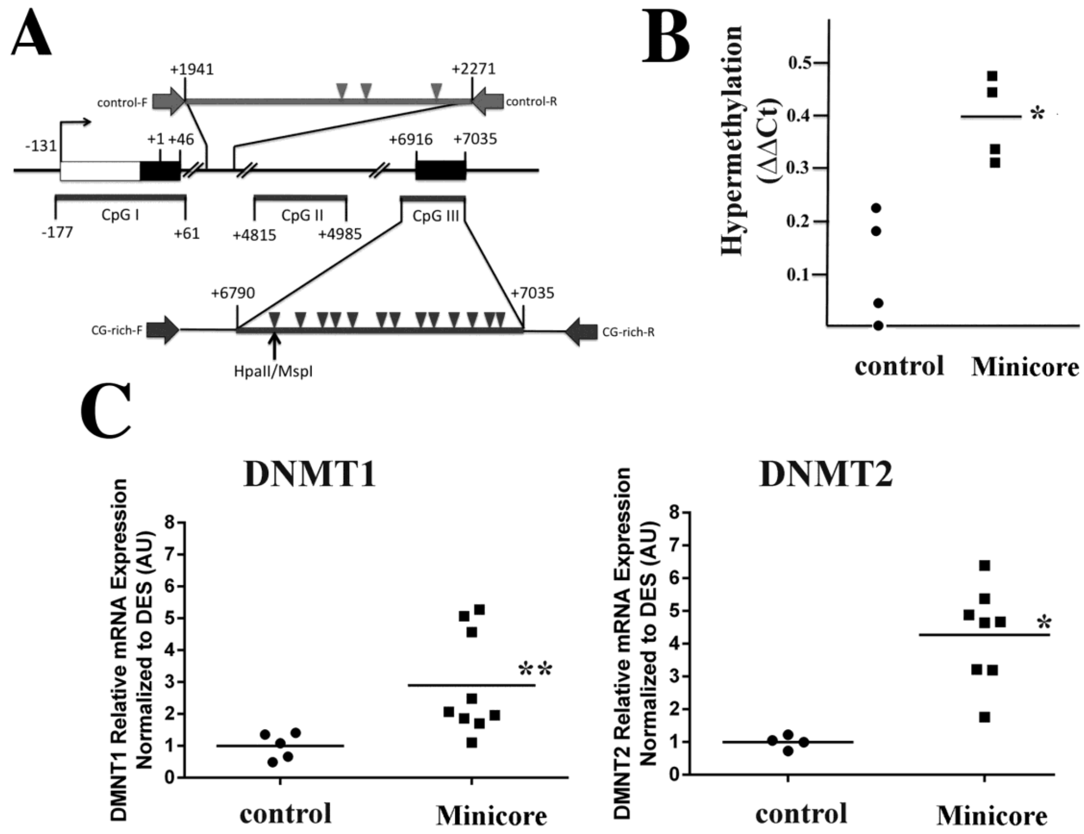

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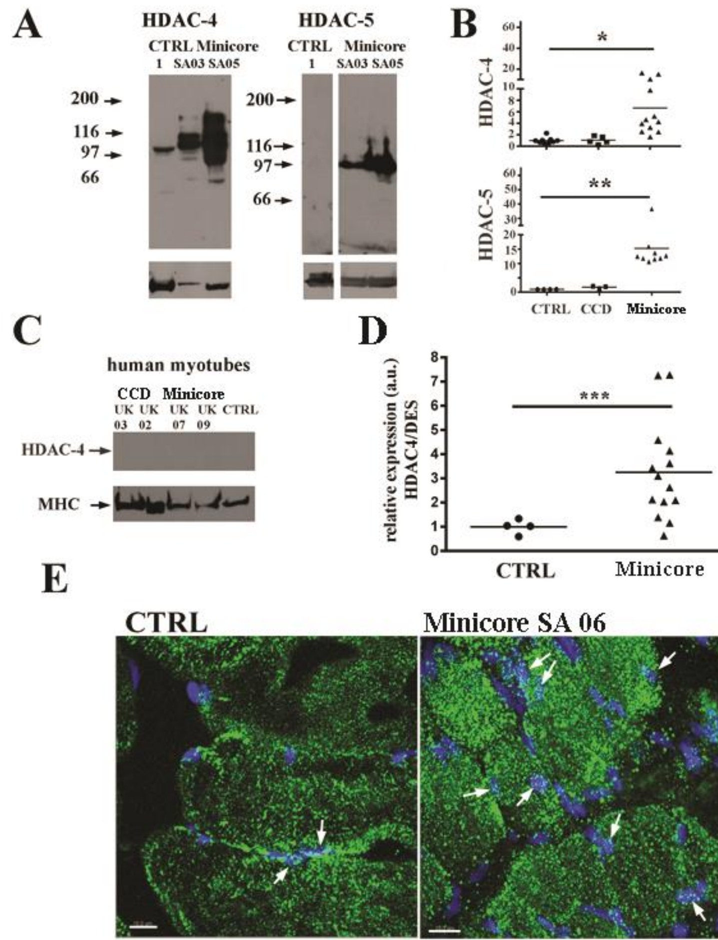

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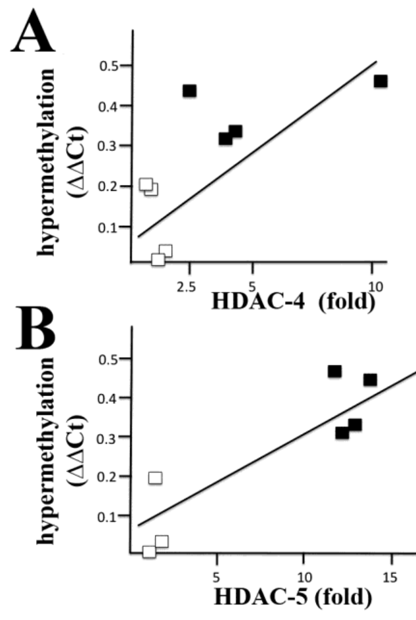

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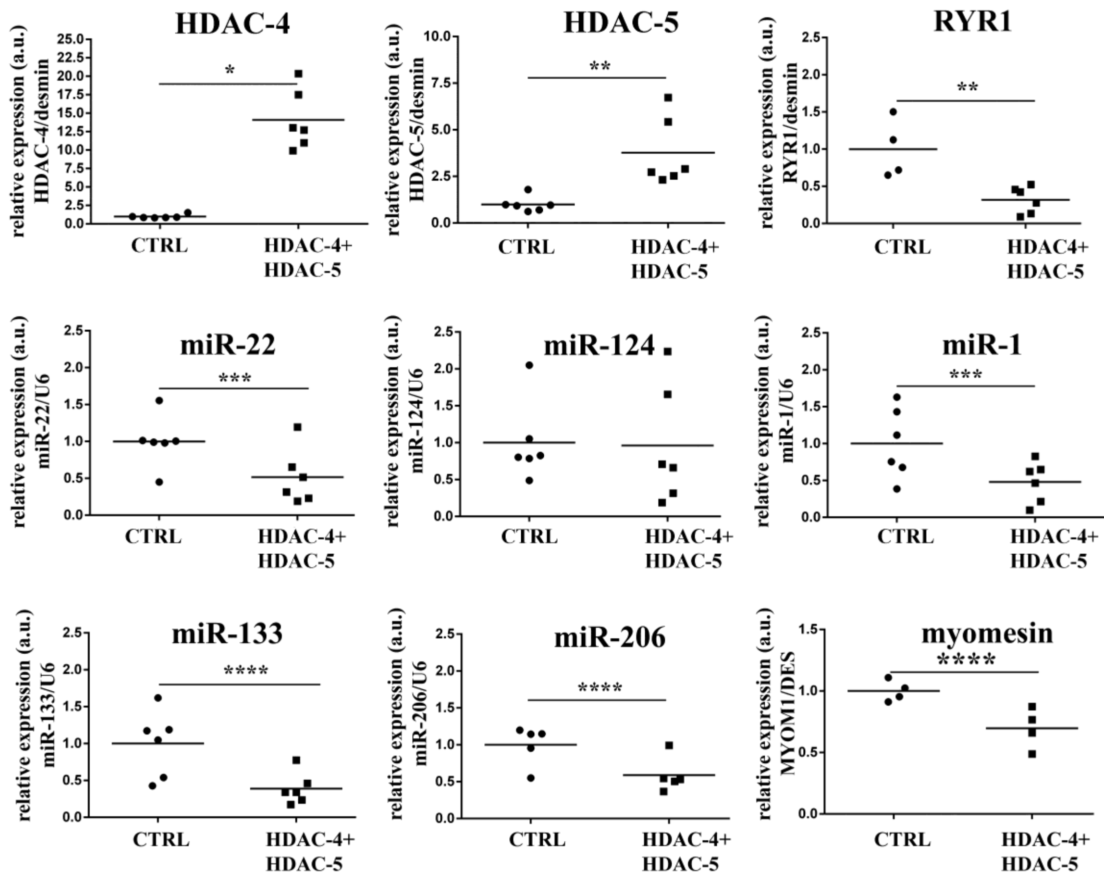

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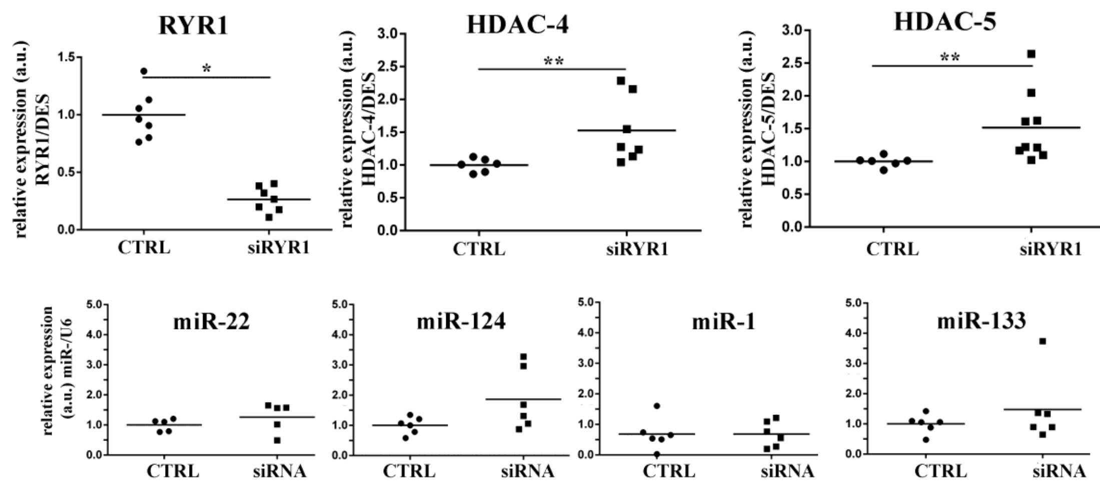

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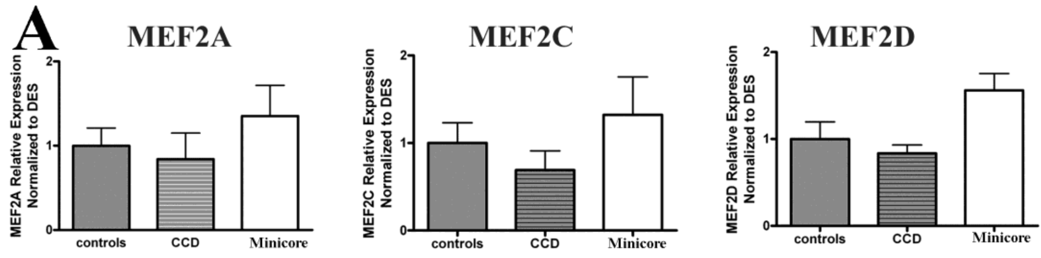

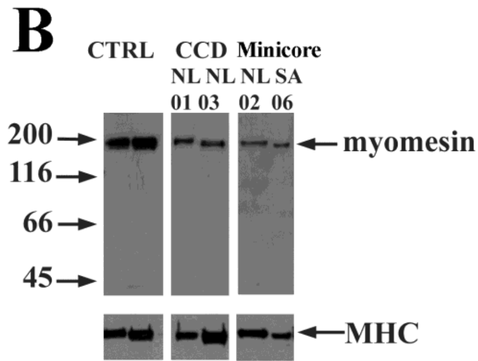

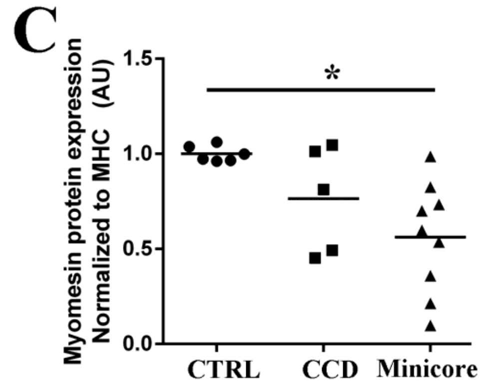

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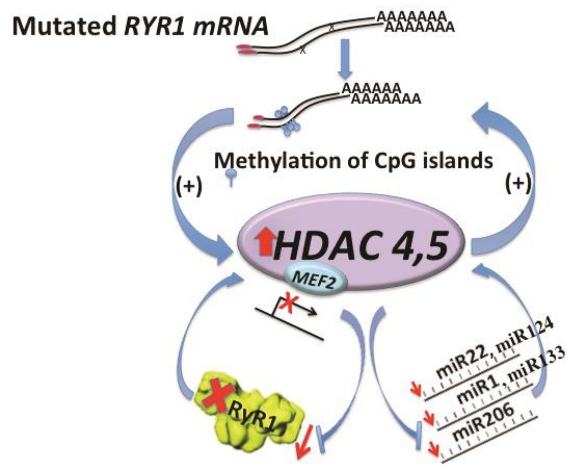

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