**Improvement of muscle strength in a mouse model for congenital myopathy treated with
HDAC and DNA methyltransferase inhibitors**

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Abstract

To date there are no therapies for patients with congenital myopathies, muscle disorders causing poor quality of life of affected individuals. In approximately 30% of the cases, patients with congenital myopathies carry either dominant or recessive mutations in the *RYR1* gene; recessive *RYR1* mutations are accompanied by reduction of RyR1 expression and content in skeletal muscles and are associated with fiber hypotrophy and muscle weakness. Importantly, muscles of patients with recessive *RYR1* mutations exhibit increased content of class II histone de-acetylases and of DNA genomic methylation. We recently created a mouse model knocked-in for the p.Q1970fsX16+p.A4329D RyR1 mutations, which are isogenic to those carried by a severely affected child suffering from a recessive form of RyR1-related multi-mini core disease. The phenotype of the RyR1 mutant mice recapitulates many aspects of the clinical picture of patients carrying recessive *RYR1* mutations. We treated the compound heterozygous mice with a combination of two drugs targeting DNA methylases and class II histone de-acetylases. Here we show that treatment of the mutant mice with drugs targeting epigenetic enzymes improves muscle strength, RyR1 protein content and muscle ultrastructure. This study provides proof of concept for the pharmacological treatment of patients with congenital myopathies linked to recessive *RYR1* mutations.

Introduction

Skeletal muscle contraction is initiated by a massive release of Ca^{2+} from the sarcoplasmic reticulum (SR) via the opening of the ryanodine receptor 1 (RyR1), a calcium release channel, which is localized in the SR terminal cisternae (1-3). The signal causing the opening of the RyR1 is the depolarization of the sarcolemmal membrane, which is sensed by voltage-dependent L-type Ca^{2+} channels (dihydropyridine receptor, DHPR) located in invaginations of the sarcolemma referred to as transverse tubules (TTs)(1, 4). Communication between DHPR and RyR1 occurs in specialized intracellular junctions between TT and SR called Ca^{2+} release units (CRUs). Skeletal muscle relaxation is brought about by SR Ca^{2+} uptake via the activity of the sarco(endo)plasmic reticulum CaATPases (SERCA)(5). Dis-regulation of Ca^{2+} signals due to defects in key proteins (RyR1 and DHPR) involved in excitation-contraction (EC) coupling is the underlying feature of several neuromuscular disorders (6-8). In particular, mutations in *RYR1*, the gene encoding RyR1, are causative of malignant hyperthermia (MH; MIM #145600), central core disease (CCD; MIM #11700), specific forms of multi-minicore disease (MmD; MIM # 255320) and centronuclear myopathy (CNM)(6-9). A great deal of data has shown that *RYR1* mutations result mainly in four types of channel defects (7). One class of mutations (dominant, MH-associated) causes the channels to become hypersensitive to activation by electrical and pharmacological stimuli (9). The second class of *RYR1* mutations (dominant, CCD-associated) results in leaky channels leading to depletion of Ca^{2+} from SR stores (7, 8). A third class of *RYR1* mutations also linked to CCD causes EC uncoupling, whereby activation of the voltage sensor $\text{Ca}_v1.1$ is unable to cause release of Ca^{2+} from the SR (10). The fourth class comprises recessive mutations, which are accompanied by a decreased content of mutant RyR1 channels on SR membranes (11-14).

67 Patients with Congenital Myopathies such as MmD carrying recessive *RYR1* mutations
68 belonging to class 4 channel defects, typically exhibit non-progressive proximal muscle
69 weakness (15, 16).

70 This reduced muscle strength is consistent with the lower RyR1 content observed in adult muscle
71 fibers that should result in a decrease of Ca^{2+} release from the SR (11-15). The decrease of RyR1
72 expression is also associated with moderate fiber atrophy, which may additionally contribute to
73 the decrease of muscle strength. In addition to the depletion of RyR1 protein, muscles of patients
74 with recessive *RYR1* mutations exhibit striking epigenetic changes, including altered expression
75 of microRNAs, an increased content of HDAC-4 and HDAC-5 and hypermethylation of more
76 than 3600 CpG genomic sites (17-19). Importantly, in muscle biopsies from 4 patients
77 hypermethylation of one of the internal *RYR1* CpG islands correlated with the increased levels of
78 HDAC-4 and HDAC-5 (18).

79 In order to study in more detail the mechanism of disease of recessive *RYR1* mutations, we
80 developed a mouse model knocked in for two mutations, isogenic to those identified in a
81 severely affected child with recessively inherited MmD (16). Such a mouse model carries the
82 p.Q1970fsX16+p.A4329D RyR1 mutations (20) and will henceforth be referred to as double
83 heterozygous (dHT). Characterization of the muscle phenotype of dHT mice demonstrated that it
84 faithfully recapitulates not only the physiological and biochemical changes, but also the major
85 muscle epigenetic signatures observed in muscle biopsies from MmD patients. In the present
86 study we treated dHT mice with drugs targeting epigenetic enzymes and evaluated the
87 physiological effects of the treatment on muscle function as well as on muscle structure. Our
88 results show that treatment of dHT mice with drugs targeting epigenetic enzymes rescues muscle
89 strength, increases RyR1 protein content and improves muscle morphology, i.e. the treatment

partially rescues Ca^{2+} release units (CRUs, the intracellular sites containing RyR1), and mitochondria. This study provides proof of concept for the treatment of patients with congenital myopathies linked to recessive *RYR1* mutations, with small molecules inhibiting DNA methyltransferases and histone deacetylases.

Results

Effect of TMP269 and 5-Aza-2-deoxycytidine on the *in vivo* muscle phenotype of dHT mice.

We first examined the pharmacokinetics and bio-distribution of TMP269, the class IIa-HDAC inhibitor we selected for this study. After intraperitoneal injection of 25 mg/kg body weight of TMP269 dissolved in Polyethylenglycol 300 (PEG300) (500 $\mu\text{l/Kg}$) and N-Methyl-2-pyrrolidone (NMP) (250 $\mu\text{L/Kg}$), blood and/or skeletal muscles were collected at different time points and the content of TMP269 was quantified by liquid chromatography tandem mass spectrometry (21). The peak blood concentration of TMP269 was achieved approximately 1 hour after injection. The circulating levels of TMP269 decay within 12 hours (Appendix 1-figure 1A). Importantly, the class II-HDAC inhibitor diffuses into skeletal muscle (Appendix 1-figure 1B), and as expected, its concentration profile in skeletal muscle follows that observed in blood. Although the level of TMP269 accumulating in skeletal muscle is lower compared to that present in blood, its concentration in muscle is adequate to induce an inhibitory effect on class IIa HDACs activity (22). An identical protocol was used to monitor the optimal dose of 5-Aza-2-deoxycytidine (5-Aza), an FDA approved DNA methyltransferase (DNMT) inhibitor (23). Administration of TMP269+5-Aza for 15 weeks resulted in hypomethylation of 165 protein-coding genes (Supplementary File 1) in soleus muscles. Gene Ontology analysis showed that most of the hypomethylated genes belong to pathways involved in gene transcription, kinase

activity and membrane targeting (Appendix 2-figure 1). Importantly, administration of TMP269+5-Aza for 15 weeks increases the acetylation of Lys residues (Appendix 2-figure 2A and Appendix 2-figure 2 source data 1-5) and of H3K9 (Appendix 2-figure 2B, 2D and Appendix 2-figure source data 1-5) in total homogenates from FDB fibers isolated from WT and dHT mice, compared to that observed in fibers from vehicle treated WT and dHT mice. This result unequivocally indicates that the inhibition of the deacetylation activity of class IIa HDACs by TMP269 occurs in the nuclei. In addition, Appendix 2-figure 2 also shows that in the absence of TMP269+5-Aza treatment, the level of H3K9 acetylation in myonuclei from *flexor digitorum brevis* (FDB) fibers isolated from dHT mice is 50% lower compared to WT. This result is consistent with: 1) higher deacetylation activity by class IIa HDACs in vehicle treated dHT mice compared to TMP269+Aza treated dHT mice and WT mice; 2) enrichment of class II HDACs in the nuclei of dHT mice. Furthermore, these results confirm that the drugs reach skeletal muscles where they exerted their biological function.

We next conducted a series of experiments to determine how the drug treatment affects *in vivo* skeletal muscle function. To this end we injected 6 weeks old WT and dHT mice with vehicle alone (PEG300+ NMP), with TMP269 alone (25 mg/kg), 5-Aza alone (0.05 mg/kg) or with the two drugs combined on a daily basis and investigated their effects on the *in vivo* skeletal muscle phenotype by analyzing forelimb grip force using a grip strength meter. Administration of each drug singly, namely TMP269 or 5-Aza alone, does not induce any change in the grip strength of WT (Fig. 1A top left and middle panels). The combined drug treatment did not affect the grip strength of WT mice (Fig. 1A, top right panel). In dHT mice, TMP269 alone causes a small but significant increase of grip strength after 10 weeks of treatment (Fig1A low left panel). However, the increased grip strength in dHT mice (Fig. 1A lower right panel) was more evident by the

combined drug treatment. In particular, this effect became apparent 4/5 weeks after starting the drug treatment and peaked at 10 weeks. The combined drug treatment rescues approximately 20% of muscle grip strength in dHT mice (see Figure 1A-figure supplement 1 for raw data on the grip strength of individual dHT mice before and after treatment with TMP269+5-Aza). Based on these results, we performed all subsequent experiments only with the combined drug treatment (i.e. TMP269+5-Aza).

We next assessed *in vivo* muscle function of WT and dHT vehicle treated or drug treated mice using the voluntary running wheel. We calculated the total running distance of WT (Fig.1B, top panels) and dHT (Fig.1B, bottom panels) mice injected with vehicle and compared it to that of mice treated for 15-18 weeks with TMP269+5-Aza. Three weeks of training improved running performance in both mouse groups. Nevertheless, on day 20 the total running distance of WT mice injected with vehicle alone was approx. 70 % greater compared to that of vehicle treated dHT mice: the total running distance of vehicle treated WT and dHT mice was 186.27 ± 16.70 km $n=4$ vs 59.93 ± 21.38 km $n=5$, respectively (mean $\pm$ S.D., Mann-Whitney two-tailed test, calculated over the 20 days of running, $*p<0.05$). On the other hand, treatment of dHT mice with TMP269+5-Aza has a remarkable effect on the total running distance (Fig. 1B, lower panel). The beneficial effect begins one week after treatment commencement, and on day 20 the total running distance achieved by dHT mice injected with TMP269+5-Aza was two times higher compared to that covered by dHT mice injected with vehicle alone (Fig. 1B lower panel; Mann-Whitney two-tailed test; on day 20, $P=0.041$): the total running distance for vehicle treated and drug treated dHT mice was 59.93 ± 21.38 km $n=5$ and 125.90 ± 16.39 km $n=5$, respectively (mean $\pm$ S.D., Mann-Whitney two-tailed test, calculated over the 20 days of running $*p<0.05$). The longer running distance was also associated with an increase median cruise speed of the

159 drug treated dHT mice compared to vehicle-treated dHT mice (Fig. 1B, right panels) (Mann-
160 Whitney two-tailed test, calculated over the 20 days running period, $*p<0.05$). The epigenetic
161 modifying drugs most likely affect a number of genes, which in turn leads to an improvement of
162 the *in vivo* muscle performance of the dHT mice; the latter effect may result from an
163 improvement of the mechanical properties of skeletal muscles and/or by an influence of the
164 drugs on the metabolic pathways of muscles. In the next set of experiments, we investigated the
165 mechanical properties of intact EDL and soleus muscles from WT and dHT mice after injection
166 of vehicle and of TMP269+5-Aza.

Figure 1: Treatment of dHT mice with TMP269+5Aza improves *in vivo* muscle function as assessed using the grip strength test and voluntary running wheel. **A.** Forelimb (2 paws) grip force measurement of WT (upper panels) and dHT (lower panels) mice treated with vehicle (WT, n= 9; dHT, n=10), TMP269 (WT, n= 11; dHT, n=6), 5-Aza (WT, n= 5, dHT, n=10) and TMP269+5-Aza (WT, n= 10, dHT, n=13). Grip strength was performed once per week during a period of 10 weeks. Each symbol represents the average ($\pm$ S.D.) grip force obtained in the indicated number (n) of mice. Grip force (Force) values obtained on the first week were considered 100%. Black symbols, vehicle treated-mice, colored symbols, drug-treated mice. Statistical analysis was conducted using the Mann-Whitney test. * $p < 0.05$. **B.** Spontaneous locomotor (dark phase) activity (left panel) and total running speed (right panel) measured over 20 days in 21-week-old dHT and WT littermates mice treated with vehicle or TMP269+5-Aza. Data points are expressed as mean ($\pm$ S.D; n=4-5 individual mice). * $p < 0.05$ (Mann-Whitney test). The exact P

Effect of TMP269+5-Aza treatment on isometric force development in muscles from WT and dHT heterozygous mice

EDL and soleus muscles isolated from mice treated for 15 weeks with vehicle or TMP269+5-Aza, were stimulated with a single 15 V pulse of 1.0 msec duration (Fig. 2A, C, E and G) or by a train of pulses delivered at 150 Hz for 400 msec (EDL, Fig. 2B and D) or 120 Hz for 1100 msec (soleus, Fig. 2F and H) to obtain maximal tetanic contracture. The averaged specific twitch peak force induced by a single action potential in EDL from dHT mice injected with vehicle alone was approximately 37% of that obtained from EDLs from WT mice (64.92 ± 13.93 mN/mm², n=10 vs $171.24 \pm 29.32^{**}$ mN/mm², n=8, respectively; mean $\pm$ S.D.; ANOVA followed

176 by the Bonferroni post hoc test $**p<0.01$. Supplementary File 2). The peak force developed after
 177 twitch stimulation of soleus muscles from dHT mice injected with vehicle alone was approx.
 178 67% of that of obtained in soleus muscles from WT littermates injected with vehicle
 179 (67.55 ± 11.26 mN/mm², n=10 vs $96.58\pm 25.78^*$ mN/mm², n=8, respectively; mean $\pm$ S.D.
 180 ANOVA followed by the Bonferroni post hoc test $*p<0.05$. Supplementary File 2). While the
 181 combined drug treatment does not affect the force developed by EDL muscles, we found that it
 182 induces a 25% increase of the twitch force in soleus muscles from dHT mice compared to that
 183 obtained from vehicle treated dHT mice (84.61 ± 14.06 mN/mm², n=13 vs $67.55\pm 11.26^*$
 184 mN/mm², n=10, respectively; mean $\pm$ S.D. ANOVA followed by the Bonferroni post hoc test
 185 $*p<0.05$. Fig. 2G and Supplementary File 2). We next examined the force developed during
 186 tetanic contractions of EDL and soleus muscles stimulated by a train of pulses delivered at 150
 187 Hz and 120 Hz, respectively. The maximal specific tetanic force developed in EDL muscles from
 188 dHT mice injected with vehicle was approximately 20% lower compared to that of EDL muscles
 189 from WT mice ($373.76\pm 73.16^*$ mN/mm², n=10 vs 452.97 ± 89.59 mN/mm², n=8, respectively;
 190 mean $\pm$ S.D. ANOVA followed by the Bonferroni post hoc test $*p <0.05$. Fig.2B and D and
 191 Supplementary File 2). We found no effect of the combined drug treatment on the maximal
 192 specific force developed by EDL muscles isolated from dHT mice (Supplementary File 2). The
 193 maximal specific tetanic force generation observed in soleus muscles from dHT mice injected
 194 with vehicle was 13% lower compared to WT ($276.29\pm 40.04^*$ mN/mm², n=10 vs 315.86 ± 56.96
 195 mN/mm², n=8, mean $\pm$ S.D. ANOVA followed by the Bonferroni post hoc test $*p<0.05$) (Fig. 2F
 196 and H). Contrary to what we observed in EDL muscles, the combined drug treatment fully
 197 rescues the maximal tetanic force of slow twitch muscles. Indeed, soleus from dHT mice treated
 198 with TMP269+5-Aza for 15 weeks displayed a maximal tetanic force which was 18% higher

compared to that of soleus from dHT mice injected with vehicle ($334.78 \pm 65.74^* \text{ mN/mm}^2$, $n=13$ vs $276.29 \pm 40.04 \text{ mN/mm}^2$, $n=10$, mean $\pm$ S.D. ANOVA followed by the Bonferroni post hoc test * $p < 0.05$) (Supplementary File 2).

Figure 2. The mechanical properties of dHT treated with TMP269+5-aza improve after 15 weeks of treatment. Mechanical properties of EDL and soleus muscle from WT and dHT mice treated with vehicle (WT, $n=8$; dHT, $n=10$) and dHT treated with TMP269+5-Aza, $n=13$). **A.** Representative traces of twitch and **B.** maximal tetanic force in EDL (150 Hz) muscle from WT and dHT. Force is expressed as Specific Force, mN/mm^2 . **C.** Statistical analysis of force generated after twitch and **D.** tetanic stimulation of isolated EDL muscle. Data points are expressed as Whisker plots ($n = 8-13$ mice). Each symbol represents the value from a muscle from a single mouse. **E.** Representative traces of twitch and **F.** maximal tetanic force (120 Hz) of soleus muscle from WT and dHT mice. **G.** Whisker plots of force generated after twitch and **H.** tetanic stimulation of isolated soleus muscles. Each symbol represents the value from a muscle from a single mouse ($n = 8-13$ mice). * $p < 0.05$ ** $p < 0.01$ (ANOVA followed by the Bonferroni post hoc test). The exact P values are given in Supplementary File

Fiber type composition and minimal Feret's diameter of soleus muscles from dHT mice treated with TMP269+5-Aza. In this set of experiments, we examined whether the ergogenic effect associated with the inhibition of epigenetic modifying enzymes is linked to fast-to-slow fiber transition and/or to changes of minimal Feret's diameter. Treatment with TMP269+5-Aza causes no changes in the content of the fiber type composition of soleus muscles (Fig. 3A and B, and Supplementary File 3). Similarly, the improved specific force cannot be attributed to a major shift of minimal Feret's fiber diameter distribution (Fig. 3C).

Figure 3: Histology of soleus muscles from TMP269+5-Aza and vehicle treated dHT mice. **A.** Analysis of soleus muscles from WT (vehicle treated) and dHT (vehicle and TMP269+5-Aza treated) mice using monoclonal antibodies specific for myosin heavy chain (MyHC) isoforms. Frozen muscle sections were stained with anti-MyHC I antibodies (slow fibers, blue), anti-MyHC IIa antibodies (fast fibers, yellow) and counterstained with anti-laminin antibodies (red). MyHCIIx (fast fibers) are unstained. **B.** Bar plots of fiber type composition of soleus muscles. Left, mean ($\pm$ S.E.M.) MyHC I fibers, middle, mean ($\pm$ S.E.M.) MyHC IIa fibers, right mean ($\pm$ S.E.M.) MyHC IIx fibers. **C.** Minimal Feret's distribution of type I and type II fibers. Data points are expressed as mean ($\pm$ S.E.M.). For WT vehicle treated a total of 2881 fibers from 3 mice were counted, for dHT vehicle treated a total of 2642 fibers from 3 mice were counted, for dHT TMP269+5-Aza a total of 2983 fibers from 3 mice were counted.

211

212 **Effect of TMP269+5-Aza treatment on calcium transients in single FDB fibers from WT**
213 **and dHT mice.** We investigated resting $[Ca^{2+}]$ and calcium transients evoked either by a single
214 pulse (Fig. 4A and B) or by a train of action potentials (Fig. 4C and D) in *flexor digitorum brevis*
215 (FDB) fibers from WT and dHT mice treated for 15 weeks with vehicle or TMP269+5-Aza (4-6
216 mice per group). We used single FDB fibers (i) because they are a mixture of fast and slow

217 twitch muscles and (ii) since intact single fibers from EDL and soleus muscles from 22 weeks
 218 old mice are nearly impossible to obtain. We found that the resting $[Ca^{2+}]$ was similar in FDB
 219 fibers from WT and dHT vehicle or drug treated mice. The Fura-2 fluorescence values
 220 (F340/F380, mean $\pm$ S.D.) were 0.81 ± 0.09 (n= 75 fibers isolated from 4 mice), 0.77 ± 0.07 (n=
 221 40 fibers isolated from 5 mice) and 0.81 ± 0.08 (n=33 fibers isolated from 5 mice) in WT mice
 222 injected with vehicle, dHT mice injected with vehicle, and dHT mice treated with TMP269+5-
 223 Aza, respectively. We next investigated the Ca^{2+} transients in response to electrical stimulation
 224 in FDB fibers loaded with 10 μ M of the low affinity Ca^{2+} indicator Mag-Fluo-4. In the presence
 225 of 1.8 mM Ca^{2+} in the extracellular solution, baseline fluorescence (mean $\pm$ SD fluorescence in
 226 arbitrary units) was similar in FDBs from vehicle treated WT mice (1.13 ± 0.12 , n=91 fibers
 227 isolated from n=4 mice), in vehicle treated dHT mice (1.14 ± 0.12 , n=110 fibers isolated from n=6
 228 mice) and drug treated dHT mice (1.16 ± 0.10 , n=155 fibers isolated from n=5 mice) (Appendix
 229 3-figure 1). In the presence of 1.8 mM Ca^{2+} in the extracellular solution, the average peak
 230 intracellular Ca^{2+} transient induced by a single action potential in FDB fibers from dHT mice
 231 injected with vehicle is approx. 30% lower than that observed in fibers from WT mice ($\Delta F/F_0$
 232 values were 0.98 ± 0.22 , n=110 fibers isolated from 6 mice vs 1.38 ± 0.30 , n=91 fibers isolated
 233 from 4 mice, respectively; mean $\pm$ S.D. Fig. 4A and B, Supplementary File 4). Interestingly,
 234 treatment of dHT mice with TMP269+5-Aza causes a 23% increase of the peak Ca^{2+} transient
 235 compared to that observed in dHT mice injected with vehicle ($*1.21 \pm 0.28$, n=155 fibers isolated
 236 from 5 mice vs 0.98 ± 0.22 , n=110 fibers isolated from 6 mice, respectively; $\Delta F/F_0$ values are
 237 expressed as mean $\pm$ S.D. ANOVA followed by the Bonferroni post hoc test $*p < 0.05$. Fig. 4A
 238 and B and Supplementary File 4). In the presence of 1.8 mM Ca^{2+} in the extracellular solution,
 239 the peak Ca^{2+} transient evoked by a train of pulses delivered at 100Hz in FDB fibers from dHT

240 mice injected with vehicle, is approx. 25% lower compared to that of FDB fibers from WT mice
241 (* 1.22 ± 0.22 , n=92 fibers isolated from 6 mice vs 1.62 ± 0.21 , n=63 fibers isolated from 4 mice,
242 respectively; $\Delta F/F_0$ values are expressed as mean $\pm$ S.D. Fig. 4D and Supplementary File 4).
243 When dHT mice are treated with TMP269+5-Aza for 15 weeks, the summation of calcium
244 transients induced by a train of supramaximal pulses, is 16% higher compared to that of dHT
245 mice injected with vehicle alone (* 1.42 ± 0.23 , n=78 fibers isolated from 5 mice vs 1.22 ± 0.22 ,
246 n=92 fibers isolated from 6 mice, respectively; $\Delta F/F_0$ values are expressed as mean $\pm$ S.D.;
247 ANOVA followed by the Bonferroni post hoc test * $p < 0.05$. Fig. 4C and D and Supplementary
248 File 4). Altogether, the increase of the peak calcium transient after either a single action potential
249 or a train of pulses is consistent, at least in part, with the ergogenic effects caused by the
250 combined treatment with TMP269+5-Aza on dHT mice.

Figure 4. Electrically evoked peak Ca^{2+} transients in muscle fibers from treated dHT compound heterozygous (dHT) mice was rescued by TMP269+5Aza administration. Enzymatically dissociated FDB fibers dissected from 4-6 mice per group, were loaded with Mag-Fluo-4 and electrically stimulated by field stimulation. Black line, vehicle treated WT, grey line, vehicle treated dHT, red line, TMP269+5Aza treated dHT.

A. Representative Ca^{2+} transient evoked by a single pulse (twitch) of 50 V with a duration of 1 msec. **B.** Whisker plots of peak twitch. Each symbol represents results obtained from a single FDB fiber. **C.** Representative Ca^{2+} transient evoked by tetanic stimulation by a train of pulses delivered at 100 Hz for 300 msec. **D.** Whisker plots of peak transient induced by tetanic stimulation.

Each symbol represents results obtained from a single FDB fiber. * $p < 0.05$ (ANOVA followed by the Bonferroni post hoc test). The exact P values are given in Supplementary File 4.

TMP269+5-Aza rescues RyR1 expression in muscles from dHT mice. The results obtained so far indicate that treatment with TMP269+5-Aza exerts a beneficial effect preferentially on slow twitch muscles. Nevertheless, the genome wide effects linked to the combined inhibition of class II HDACs and DNMT may affect a number of processes underlying muscle strength, making it difficult if not impossible to dissect the exact mechanisms underlying the improvement of slow twitch muscle function observed in dHT mice. However, based on our previous results, we postulate that the improvement of muscle strength observed in soleus muscles may be explained, at least in part, by an increase of the key proteins involved in skeletal muscle activation. Fig. 5A

shows *Ryr1* transcript expression in soleus muscles from WT and dHT mice. Treatment with vehicle alone does not rescue *Ryr1* expression (Mann-Whitney two-tailed test, WT vehicle vs dHT vehicle, $P=0.039$), however treatment with TMP269+5-Aza for 15 weeks causes a significant increase in *Ryr1* transcript levels (Mann-Whitney two-tailed test, dHT vehicle vs dHT TMP269+5-Aza, $P=0.019$). *Cacna1s* levels are not affected by vehicle or drug treatment. *Hdac4* transcript levels are increased in soleus muscles from dHT vehicle treated mice, compared to vehicle treated WT mice (Fig. 5A, Mann-Whitney two-tailed test, WT vehicle vs dHT vehicle, $P=0.041$). *Hdac4* transcript levels decrease in muscles from TMP269+5-Aza treated dHT mice compared to vehicle treated dHT mice (Fig. 5A, Mann-Whitney two-tailed test, dHT vehicle vs dHT TMP269+5-Aza, $P=0.002$). We also investigated RyR1 protein content in total homogenates from soleus muscles from WT and dHT mice. The RyR1 protein content of soleus muscles from dHT mice injected with vehicle is 46% lower compared to that of WT mice (Fig. 5B and Figure 5B source data 1-3). The mean $\pm$ S.D. % intensity of the immunopositive band corresponding to RyR1 is $100\% \pm 7.30$, $n=8$ in WT vs $54.93\% \pm 18.45$, $n=6$ in dHT, respectively (ANOVA followed by the Bonferroni post hoc test, $*P=0.039$). The RyR1 protein content is partially re-established by treatment with TMP269+5-Aza. Indeed, the RyR1 protein content in drug treated dHT mice increased and reaches a value of $88.03\% \pm 10.42^*$, $n=7$, in contrast to the $54.93\% \pm 18.45$, $n=6$ found in vehicle treated dHT mice (ANOVA followed by the Bonferroni post hoc test, $*P=0.037$ for vehicle vs TMP269+5-Aza treated dHT mice; Fig. 5B and Fig.5B source data 1-3). This recovery of RyR1 is consistent with the *in vivo* and *in vitro* muscle phenotype amelioration induced by the combined drug treatment. We also measured the effect of the combined drug treatment on the content of other proteins involved in skeletal muscle ECC

including SERCA1, SERCA2, calsequestrin 1 and JP-45. As shown in Fig. 5C (and Fig. 5C source data 1-8) the muscle content of these proteins is unaffected by the drug treatment.

Figure 5. Treatment with TMP269+5Aza reverses RyR1 loss in soleus muscles from dHT mice. **A.** Real time qPCR on RNA isolated from soleus muscles isolated from vehicle treated WT and vehicle treated and TMP269+5-Aza treated dHT mice. Experiments were carried out on muscles isolated from 4 mice per group. RNA isolation and amplification conditions as described in the Methods section. *p<0.05; **p<0.01 ANOVA followed by the Bonferoni post hoc test. **B.** Western blot analysis of RyR1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 6 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: representative images of blot probed with anti-RyR1 Ab followed by anti-MyHC Ab for normalization. Right panel, data points are expressed as Whisker plots *p<0.05 (ANOVA followed by the Bonferoni post hoc test). **C** (left panel) representative immunoblots of total homogenates soleus muscles from WT (vehicle) and dHT (vehicle and TMP269+5Aza) mice probed with the indicated antibodies. (Right panel) bar histograms showing the mean ± S.D. (n=4 mice per group) intensity of the immunopositive band, expressed as % of the intensity of the band in WT (vehicle treated) mice. MyHC was used for loading protein normalization. Forty µg of protein were loaded per lane and proteins were separated on 7.5% or 10% SDS-PAG. Exact P values are given in the

Recovery of calcium release units (CRUs) in soleus muscles from dHT after TMP269+5-Aza treatment. Skeletal muscle fibers from adult wild type (WT) are usually characterized by a regular transverse pale-dark striations. Within the fiber interior Ca release units (CRUs), the SR-TT junctions containing RyR1, are uniformly distributed, and mostly placed at A-I band transition (when sarcomeres are relaxed), on both sides of the Z line. CRUs are formed by two SR terminal cisternae closely opposed to a central TT oriented transversally with respect to the

longitudinal axis of the myofibrils. These CRUs are called triads. CRUs are often associated with a mitochondrion, to form functional couples (24).

In soleus muscle fibers from vehicle treated dHT mice (Fig. 6A and B) CRUs present some abnormal features: the SR is often dilated (small arrows in Fig. 6A) and at times they are incomplete, meaning they are formed by only 2 elements (dyads) (Fig. 6B, black arrow).

Quantitative EM analysis shows that in fibers of vehicle treated dHT mice there are 40.3 ± 2.4 CRUs/ $100 \mu\text{m}^2$, $14.2 \pm 3.6\%$ of them being dyads (Table 1, columns A-B). In soleus muscle fibers of dHT vehicle treated mice there are also regions with accumulated autophagic material (Fig. 6B, empty arrow). In fibers from TMP269+5-Aza treated dHT mice (Fig. 6C and D), analysis of CRUs indicates a (at least partial) rescue of the EC coupling machinery. Dilated SR in triads (pointed by small arrows in Fig. 6A) are practically absent (Fig. 6C), and CRUs are more abundant and better preserved, meaning that they have the classic triad structure (3 elements: 2 SR and one T-tubule, small arrows in Fig. 6D). Quantitative analysis indicates that there are 46.9 ± 2.3 CRUs/ $100 \mu\text{m}^2$, only $6.5 \pm 0.6\%$ of them being dyads (Table 1, columns A-B).

We also analyzed mitochondria and their association with CRUs (Table 2). In TMP269+5-Aza treated dHT versus vehicle treated mice: i) the number/area of mitochondria is significantly increased (71.0 ± 3.5 v.s 59.7 ± 2.9 mean $\pm$ S.E.M; Table 2, column A); ii) the number of damaged mitochondria is slightly but significantly reduced (2.9 ± 0.6 vs. 3.2 ± 0.5 mean $\pm$ S.E.M; Table 2, column B), and finally iii) mitochondria are more often correctly associated with CRUs to form functional couples (34.5 ± 2.3 vs. 26.9 ± 2.2 mean $\pm$ S.E.M; Table 2, column C).

Table 1. Quantitative analysis of CRUs. In soleus fibres from TMP269+5-Aza treated dHT mice, frequency of total CRUs and dyads (incomplete CRUs) are significantly rescued (columns A and B), suggesting a better preservation of CRUs and improved structure of triads.

	A	B	C
	n. of CRUs /100 μm^2	% of Dyads	% of oblique longitudinal CRUs
SOLEUS Vehicle treated	40.3 $\pm$ 2.4	14.2 $\pm$ 3.6	10.7 $\pm$ 2.0
SOLEUS TMP269+5-Aza treated	*46.9 $\pm$ 2.3	#6.5 $\pm$ 0.6	10.6 $\pm$ 2.6

Data are shown as mean $\pm$ SEM.; n= 2 vehicle treated soleus muscles; n=3 drug treated soleus muscle

*p=0.0248

#p=0.0001

Table 2. Quantitative analysis of mitochondria. Soleus fibres from TMP269+5-Aza treated dHT mice show a significant improvement in frequency, disposition and morphology of mitochondria.

	A	B	C
	n. of mitochondria in 100 μm^2	n. of severely altered mitochondria in 100 μm^2 (%)	n. of mitochondria /CRU pairs in 100 μm^2
SOLEUS Vehicle Treated	59.7 $\pm$ 2.9	3.2 $\pm$ 0.5 (6.2)	26.9 $\pm$ 2.2
SOLEUS Drug Treated	* 71.0 $\pm$ 3.5	#2.9 $\pm$ 0.6 (3.7)	§34.5 $\pm$ 2.3

Data are shown as mean $\pm$ SEM.; n= 2 vehicle treated soleus muscles; n=3 drug treated soleus muscles.

*p =0.0175

#p=0.0475

§p=0.0201

Figure 6. Representative EM images of soleus fibres from vehicle treated (A and B) and TMP269+5-Aza treated (C and D) dHT mice. A. Representative EM images at low magnification of soleus fibres from vehicle treated and **C.** TMP269+5-Aza treated dHT mice: small arrows point to dilated SR. **B.** and **D.** Higher magnification images showing calcium release units (CRUs, black arrow) and autophagic material (empty arrow, panel B) from vehicle treated and drug treated (D) dHT mice. M = mitochondria. Scale bars: A and C, 1 µm; B and D, 500 nm.

Discussion

Congenital myopathies are rare neuromuscular disorders, caused in approximately 30% of the patients, by mutations in the *RYR1* gene (6, 8). Patients can present a variety of symptoms and phenotypes depending on whether the mutations are dominantly or recessively inherited. In particular, patients with recessive *RYR1* mutations often display involvement of extraocular muscles leading to ophthalmoplegia and or ptosis as well as involvement of respiratory muscles, often requiring assisted ventilation (6-8, 11, 15). Children may also present physical abnormalities including club foot, scoliosis, facial dysmorphisms, winged scapula and/or pectus excavatum (15). Nevertheless, the most disturbing symptom present at infancy in children carrying recessive *RYR1* mutations are hypotonia and proximal muscle weakness. In the most

severe cases, muscle weakness also impairs masticatory muscles causing dysphagia, whereby the affected infants are fed via percutaneous endoscopy gastrostomy, a procedure which influences the quality of life of the affected individuals and his/her family members. To date there are no therapies available for congenital myopathies.

In order to target these unmet clinical needs we exploited the heterozygous mouse model we created carrying compound heterozygous RyR1 mutations (p.Q1970fsX16+p.A4329D), for a preclinical study aimed at developing a therapeutic strategy for patients with congenital myopathies. In particular, we tested small molecules targeting epigenetic modifying enzymes such as class II HDACs and DNMT on male mice. The reason for performing experiments only on male mice is twofold: (i) their muscle mass is generally greater than that of females and (ii) we wanted to avoid variability linked to hormonal fluctuations. Though, the cross sectional area of fibers is larger in males than in females the fiber type composition is similar (25), thus we are confident that the beneficial effects of the treatment with TMP269+5-Aza we observed on muscle function will occur in male and female mice alike.. Here we show that the combined treatment with TMP269 and 5-Aza-2 deoxycytidine have strong ergogenic effects on muscle functions, tested both *in vivo* and *in vitro*. The improvement of muscle performance is consistent with the increase of both peak calcium transients and RyR1 protein content in total muscle homogenates and is apparently not associated with changes in the fiber type composition or changes of the minimal Feret's diameter of muscle fibers. We are confident that this study provides proof of concept for a therapeutic strategy aimed to enhance muscle strength in patients affected by congenital myopathies linked to recessive *RYR1* mutations.

Our data shows that the improvement of muscle performance *in vivo* is mainly due to the amelioration of muscle strength in slow twitch fibers. The muscle ultrastructure demonstrates a

376 partial rescue of the EC coupling machinery (more triads, less of them being incomplete) and of
377 the associated mitochondria and may explain in part the rescue of muscle function. The
378 elucidation of the exact molecular mechanisms underlying the improvement in strength
379 specifically of slow twitch fibers caused by genome wide effects of drugs inhibiting epigenetic
380 modifying enzymes is an arduous challenge. However, we believe that a few assumptions can be
381 taken into account to understand our data. First, histopathology of muscle biopsies from patients
382 harboring *RYR1*-related congenital myopathies show fiber type I predominance (8,15, 26-28).
383 Thus, the recovery of muscle function after pharmacological treatment should occur to a greater
384 extent in slow twitch fibers compared to fast twitch fibers, since the latter types of fibers are
385 mostly absent. Increasing the strength of slow twitch fibers would greatly benefit patients.
386 Furthermore, this interpretation of the histopathology of human skeletal muscle biopsies is
387 consistent with our previous results on the dHT mouse model (20). Indeed, we found that the
388 RyR1 array disarrangements and morphological alterations are more noticeable in fast glycolytic
389 muscles such as EDLs compared to soleus muscles, a result in line with the idea that skeletal
390 muscle oxidative fibers are more resistant to the damaging effects linked to the expression of
391 recessive *RYR1* mutations (20). If this is the case, then upon pharmacological treatment slow
392 oxidative fibers should recover their function to a greater extent than fast twitch glycolytic fibers.
393 We found that HDAC4 transcript levels in soleus muscles from dHT mice treated with a
394 combination of TMP269+5-Aza decreases to a value similar to that found in WT littermates.
395 Though we don't have a clear explanation for this observation, it appears to be in line with the
396 proposal that high levels of class II HDACs are indicative of muscle damage or age-related
397 atrophy and reduced expression of class II HDACs is associated with an increased running
398 endurance (29, 30). Second, it was recently demonstrated that pharmacological inhibition of

class II HDACs causes de-acetylation not only of nuclear histones but also of other targets, including MyHC and PGC1alpha (31), an event which causes an increase of the cytoplasmic level of the PGC1alpha protein. Interestingly, PGC1alpha is a crucial regulator of oxidative metabolism and mitochondrial biogenesis in skeletal muscle fibers (32). We found that administration of class II HDAC and DNMT inhibitors to dHT mice induces an increase in the number of mitochondria in slow twitch fibers. This result is in agreement with the observed increase in PGC1alpha activity observed after treatment with class II specific HDAC inhibitors (31). Third, reconstitution of the RyR1 arrays in the junctional SR might restore the RyR1 retrograde signal, which is important for the activation of $\text{Ca}_v1.1 \text{ Ca}^{2+}$ currents across T tubular membranes. Since the $\text{Ca}_v1.1$ to RyR1 ratio is lower in slow twitch muscles (33), reactivation of the $\text{Ca}_v1.1$ calcium current might occur to a larger extent in slow twitch muscles from dHT treated mice compared to fast twitch muscles. Though $\text{Ca}_v1.1$ calcium currents are not relevant for skeletal muscle EC coupling, they have been implicated in a broad range of muscle functions, including fatigue resistance, protein synthesis and calcium dependent signaling pathways involved in the maintenance of proper skeletal muscle function (34).

In conclusion, the present study demonstrates that the combined pharmacological treatment with an FDA approved DNMT inhibitor and TMP269 improves muscle strength and performance in a mouse model for recessive *RYR1* congenital myopathy. Further studies are needed to define the exact mechanism of the ergogenic effects of these genome wide epigenetic modifying drugs; nevertheless, our results provide the proof of concept for the development for pharmacological treatment of patients with congenital myopathies linked to recessive *RYR1* mutations.

Materials and Methods

Compliance with Ethical standards: All experiments involving animals were carried out on 16-21 weeks old male mice unless otherwise stated. Experimental procedures were approved by the Cantonal Veterinary Authority of Basel Stadt (BS Kantonales Veterinäramt Permit numbers 1728 and 2950). All experiments were performed in accordance with ARRIVE guidelines and Basel Stadt Cantonal regulations.

Drug injection protocol: The class II HDAC inhibitor TMP269 was purchased from Selleckchem (S7324), the DNA methyltransferase inhibitor 5-aza-2 deoxycytidine was from Sigma-Aldrich (A3656), Polyethylenglycol 300 (PEG300) was from Merck (8.17019), N-Methyl-2-pyrrolidone (NMP) was from Sigma-Aldrich (328634). Intraperitoneal injections (IP, 30-gauge needle) started at the sixth week of age and continued daily for 10 - 15 weeks. Mice received vehicle (PEG300 and NMP), TMP269 (25 mg/Kg), 5-aza-2-deoxycytidine (0.05 mg/Kg), a combination of the two compounds (TMP26+5-aza-2-deoxycytidine, 25 mg/Kg and 0.05 mg/Kg, respectively). TMP269 and 5 aza-2-deoxycytidine were diluted in PEG300 (500 µl/Kg) and NMP (250 µl/Kg). The final volume of drug or vehicle injected per mouse was 750 µl per kilogram body weight.

Pharmacokinetics analysis of TMP269: Six weeks old mice were injected intraperitoneally with 25 mg/kg bodyweight (n=4) TMP269 and blood and muscle tissue were collected at different time points post injection and analyzed. Blood (20 µL) was collected from the tail vein using lithium heparin coated capillaries (Minivette POCT 20 µL LH, Sarstedt, Germany). Samples were collected prior treatment (T0) and 1, 3, 6, 12, 24, 48 hours after TMP269 injection, transferred directly into autosampler tubes, and kept at -20°C until analyzed. For skeletal muscle samples, the following protocol was used. At T=0 and at different time points after TMP269

injection (1, 3, 12, 24, 48 hours), mice were sacrificed and their skeletal muscles were isolated, flash frozen and stored in liquid nitrogen. On the day of the analysis, muscle samples were homogenized using a tissue homogenizer (Mikro-Dismembrator S, Sartorius, Aubagne, France) for two sequences of 30 sec each.

The levels of TMP269 in blood and in skeletal muscle were quantified by high pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) as described (21). Briefly, A Shimadzu LC system (Kyoto, Japan) was used, TMP269 and TMP195 (internal standard, IS) were analyzed by positive electrospray ionization and multiple reaction monitoring (MRM). Methanol containing IS (5 nM TMP195) was used to extract TMP269 from blood and muscle homogenate samples (20 μ L blood/muscle plus 175 μ L methanol). The samples were vortex mixed for approximately 30 seconds and centrifuged at 10°C, 3220 g for 30 minutes. Ten μ L of supernatant were injected into the LC-MS/MS system. Calibration lines were prepared in blank mouse blood and covered a range of concentrations, from 0.25 nM to 500 nM TMP269. A linear regression between TMP269 to the IS peak area ratio and the nominal concentration was established with a weighting of 1/x² to quantify the TMP269 concentration in blood or muscle. The Analyst 1.6.2 software (AB Sciex, Concord, Canada) was used to operate the LC-MS/MS system and to analyze the data.

Genotyping dHT mice and real time qPCR: Mouse genotyping was performed on mouse genomic DNA, by PCR amplification of *Ryr1* exon 36 and exon 91 using specifically designed primers (Supplementary File 5), as previously described (20). Quantitative real-time polymerase chain reaction (qPCR) was carried out on RNA extracted from muscles isolated from 21 weeks old mice (treated with drug or vehicle for 15 weeks, starting at 6 weeks of age). Briefly, total RNA extracted from frozen hind limb muscles was isolated, genomic DNA was removed by

467 DNase I treatment (Invitrogen; 18068-015) and 1000 ng RNA were reverse-transcribed into
468 cDNA using the High capacity cDNA Reverse Transcription Kit (Applied Biosystems; 4368814)
469 as previously described (20). The cDNA was amplified by qPCR using the primers listed in
470 Supplementary File 5 and transcript levels were quantified using Power SYBR® Green reagent
471 Master Mix (Applied Biosystems; 4367659), using the Applied Biosystems 7500 Fast Real-time
472 PCR System running 7500 software version 2.3 as previously described (20). Transcript
473 quantification was based on the comparative $\Delta\Delta C_t$ method. Each reaction was performed in
474 duplicate and averaged and results are expressed as relative gene expression normalized to the
475 housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh).

476 ***In Vivo* Muscle Strength Assessment:** *In vivo* muscle performance was evaluated in male mice,
477 by performing the following measurements: (i) forelimb grip strength and (ii) spontaneous
478 locomotor activity. Forelimb grip strength was assessed once per week for a period of 10 weeks
479 using a Grip Strength Meter from Columbus Instruments (Columbus, OH, USA), following the
480 manufacturer's recommendations. The grip force value obtained per mouse was calculated by
481 averaging the average value of 5 measurements obtained on the same day on the same mouse.
482 To avoid experimental bias during measurements of grip force, experimenters were blinded to
483 the genotype and treatment of mice. For spontaneous locomotor activity, after 15 weeks of
484 treatment with vehicle or TMP269+ 5-Aza, mice were individually housed in cages equipped
485 with a running wheel carrying a magnet as previously described (20, 35). Wheel revolutions
486 were registered by reed sensors connected to an I-7053D Digital-Input module (Spectra), and the
487 revolution counters were read by a standard laptop computer via an I-7520 RS-485-to-RS-232
488 interface converter (Spectra). Digitized signals were processed by the "mouse running"

software developed at Santhera Pharmaceuticals (36). Total running distance (kilometer) and speed (Km/h) were evaluated.

***Ex vivo* Muscle Strength Assessment:** To test muscle force *ex vivo*, *extensor digitorum longus* (EDL) and soleus muscles were dissected from 21 weeks old male WT and dHT mice, after 15 weeks of treatment with vehicle alone, or with the combination of TMP26915-Aza. Isolated EDL and soleus muscles were mounted onto a muscle force transducing setup (MyoTronic Heidelberg) as previously described (35). Muscle force was digitized at 4 kHz by using an AD Instrument converter and stimulated with 15 V pulses for 1.0 msec. Tetanus was recorded in response to a train of pulses of 400 msec and 1100 msec duration delivered at 10/20/50/100/150 Hz, and 10/20/50/100/120 Hz, for EDL and soleus, respectively. Specific force was normalized to the muscle cross-sectional area [CSA_wet weight (mg)/length (mm)_1.06 (density mg/mm³)] (37). The experimenter performing the measurements was blinded with respect to the mouse genotype and treatment.

Isolation of single *flexor digitorum brevis* (FDB) fibers for intracellular Ca²⁺ measurements: Twenty-one weeks old WT and dHT vehicle and drug treated male mice were killed by pentobarbital overdose according to the procedures approved by the Kantonal Veterinary Authority. *Flexor digitorum brevis* (FDB) muscles were isolated and digested with 0.2% of Collagenase type I (*Clostridium hystoliticum* Type I, Sigma-Aldrich) and 0.2% of Collagenase type II (*Clostridium hystoliticum* Type II, Worthington) in Tyrode's buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.1% glucose, 11.8 mM HEPES, pH 7.4 NaOH) for 45 minutes at 37 °C as described (38). Muscles were washed with Tyrode's buffer to block the collagenase activity and gently separated from tendons using large to narrowest set of fire-polished Pasteur pipettes. Fibers obtained by this procedure remained excitable and contracted

briskly when assayed experimentally. Finally, fibers were placed on laminin coated (5 μ l of 1 mg/ml mouse laminin from ThermoFischer) 35 mm glass bottom dishes (MatTek corporation) for measurements of the resting $[Ca^{2+}]$ or on ibiTreat 15 μ -Slide 4 well (Ibidi) for electrically evoked Ca^{2+} measurements as previously described (20).

Measurements of resting $[Ca^{2+}]$ and of electrically evoked Ca^{2+} transients: Single FDB fibers were isolated from 4-5 male mice per group and allowed to adhere to laminin treated 35 mm glass bottom dishes for 1 hr at 37°C. The fibers were then loaded with 5 μ M Fura-2 AM (Invitrogen) by incubating them for 20 min at 19°C in Tyrode's buffer. The excess Fura-2 was diluted out by the addition of fresh Ringer's solution and measurements of the resting $[Ca^{2+}]$ were carried out using an inverted Zeiss Axiovert fluorescent microscope, as previously described (20, 35). Only those fibers that contracted when an electrical stimulus was applied were used for the $[Ca^{2+}]$ measurements.

For electrically evoked Ca^{2+} transients, single FDB fibers were incubated for 10 min at 19 °C in Tyrode's solution containing 10 μ M low affinity calcium indicator Mag-Fluo-4 AM (Thermo Fischer), 50 μ M N-benzyl-p-toluene sulfonamide (BTS, Tocris). Fibers were rinsed twice with fresh Tyrode's solution, and measurements were carried out in Tyrode's solution containing 50 μ M BTS. Measurements were carried out with a Nikon Eclipse inverted fluorescent microscope equipped with a 20x PH1 DL magnification objective. The light signals originating from a spot of 1 mm diameter of the magnified image of FDB fibers were converted into electrical signals by a photomultiplier (Myotronic Heidelberg). Fibers were excited at 480 nm and then stimulated either with a single pulse of 50 V with a duration of 1 msec, or with a train of pulses of 50 V with a duration of 300 msec delivered at 100 Hz. Fluorescent signals were acquired using PowerLab Chart7. Changes in fluorescence were calculated as $\Delta F/F_0 = (F_{max} - F_{rest}) / (F_{rest})$.

Kinetic parameters were analyzed using Chart7 software. FDB fibers were isolated from 4-6 mice per group and results were averaged.

Biochemical analysis of total muscle homogenates: Total muscle homogenates were prepared from soleus muscles isolated from WT and dHT vehicle and drug treated mice. SDS-polyacrylamide electrophoresis and Western blots of total homogenates were carried out as previously described (20, 35, 39). Western blots were stained with the primary antibodies listed in Supplementary File 6, followed by peroxidase-conjugated Protein G (Sigma P8170; 1:130'000) or peroxidase-conjugated anti-mouse IgG (Fab Specific) Ab (Sigma A2304; 1:200'000). The immuno-positive bands were visualized by chemiluminescence using the WesternBright ECL- HRP Substrate (Witec AG). Densitometry of the immune-positive bands was carried out using the Fusion Solo S (Witec AG).

Genome-wide DNA Methylation Analysis: Sodium-bisulfite treated DNA of nine samples (6 dHT vehicle treated and 3 dHT drug treated) was subjected to measure global DNAm by *Infinium Mouse methylation* BeadChip array (Illumina) according to manufacturer's instructions. Illumina mouse array contains ~285,000 methylation sites per sample at single-nucleotide resolution covering over 20 design categories including gene promoters and enhancers. Downstream analysis were conducted using annotation developed previously mapping to mm10. Raw IDAT files were preprocessed using R/Bioconductor package SeSAMe (40). Samples were normalized using "noob" including background subtraction and dye-bias normalization. Methylation levels were computed as Beta (β) values ($\text{Meth} / [\text{Meth} + \text{Unmeth} + 100]$) and as logit-transformed M-value ($\log_2 [\text{Meth} / \text{Unmeth}]$). Probes where at least one sample had a detection P value > 0.05 and the probes recommended previously using mm10 genome as general-purpose masking probes were filtered out (40). The remaining 215,717 CpGs were used

for further analysis. Statistical analyses were performed on M-values (41) using bioconductor *limma*, whereas β -values were used for biologic interpretation. Normalized methylation levels were evaluated using linear regression model between dHT treatment vs. vehicle. Differentially methylated CpGs were identified using empirical Bayes moderated t-statistics and associated Benjamini-Hochberg (BH) false discovery rate (FDR) adjusted *P*-values <0.05 were used as cutoff.

Histological examination: Soleus muscles from treated and untreated WT and dHT mice were isolated and mounted for fluorescence microscopy imaging. Muscles were embedded in OCT and deep-frozen in 2-Methylbutane, then stored at minus 80°C. Subsequently, transversal 10 μ m sections were obtained using a Leica Cryostat (CM1950) starting from the belly of the muscles. Sections were stained as described by Delenize et al. (42) using the following primary antibodies (see Supplementary File 6 for suppliers and catalog numbers): mouse IgG2b anti- MyHC I (1:50), mouse IgG1 anti-MyHC IIa (1:200), mouse IgM anti- MyHC IIb (1:100) and rabbit anti-mouse laminin (1:1500), followed by incubation with the following secondary antibodies: goat anti-mouse IgG1Alexa Fluor 568 (1:1000), goat anti-mouse IgM Alexa Fluor 488 (1:1000), goat anti-mouse IgG Fc γ 2b Dylight 405 (1:400) and Donkey anti-rabbit IgG Alexa Fluor 647 (1:2000). Images were obtained using an Eclipse Ti2 Nikon Fluorescence microscope with 10X air objective lens. Muscles from 3 mice per group were evaluated. Images were analyzed using Fiji plugins developed by Delenize et al. (42) in order to obtain information on fiber types and minimal Feret's diameter (36), the closest possible distance between the two parallel tangents of an object, using a combination of cell segmentation and intensity thresholds as described (36).

Preparation and quantitative analysis of samples by electron microscopy (EM). Soleus muscles were dissected from sacrificed animals, pinned on a Sylgard dish, fixed at room

temperature with 3.5% glutaraldehyde in 0.1 M NaCaCO buffer (pH 7.4), and stored in the fixative at 4°C (43). Fixed muscle were then post-fixed in a mixture of 2% OsO₄ and 0.8% K₃Fe(CN)₆ for 1-2 h, rinsed with 0.1M sodium cacodylate buffer with 75 mM CaCl₂, en-block stained with saturated uranyl acetate replacement, and embedded for EM in epoxy resin (Epon 812). Ultrathin sections (~40 nm) were cut in a Leica Ultracut R microtome (Leica Microsystem, Austria) using a Diatome diamond knife (DiatomeLtd. CH-2501 Biel, Switzerland) and examined at 60 kV after double-staining with uranyl acetate replacement and lead citrate, with a FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic), equipped with Megaview III digital camera (Munster, Germany) and Soft Imaging System (Germany).

Quantitative analyses by EM. Data contained in Tables 1 and 2 were collected on soleus muscles from 21 weeks old dHT mice, either vehicle or TMP269+5-Aza treated. In each sample, 10-20 fibers were analyzed. In each fiber 2-3 micrographs (all at the same magnification, 14K, and of non-overlapping regions) were randomly collected from longitudinal sections.

Number of CRUs (Table 1, column A), number of mitochondria, number of severely altered mitochondria, and number of mitochondrion-CRU pairs (Table 2, columns A-C, respectively) were reported as average number/100 μm^2 (24). In each EM image, we also determined the number of dyads, i.e. incomplete triads (Table 1, column B), and of oblique/longitudinal CRUs (Table 1, column C), and expressed as percentages over the total number of CRUs. Mean and SEM were determined using GraphPad Prism (GraphPad Software, San Diego, California USA). Statistically significant differences between groups were determined by the Student's *t* test (GraphPad Software, San Diego, California USA) or by a Chi-squared test (GraphPad Software, San Diego, California USA). Values of $p < 0.05$ were considered significant.

Statistical analysis. Statistical analysis was performed using the Student's unpaired *t* test for normally distributed values when two groups were being compared, or the ANOVA test followed by the Bonferroni post hoc test for multiple group comparisons. The Mann–Whitney U test was used when values were not normally distributed. $p < 0.05$ was considered significant.

Figure Legends:

Figure 1: Treatment of dHT mice with TMP269+5Aza improves *in vivo* muscle function as assessed using the grip strength test and voluntary running wheel. **A.** Forelimb (2 paws) grip force measurement of WT (upper panels) and dHT (lower panels) mice treated with vehicle (WT, $n = 9$; dHT, $n = 10$), TMP269 (WT, $n = 11$; dHT, $n = 6$), 5-Aza (WT, $n = 5$, dHT, $n = 10$) and TMP269+5-Aza (WT, $n = 10$, dHT, $n = 13$). Grip strength was performed once per week during a period of 10 weeks. Each symbol represents the average ($\pm$ S.D.) grip force obtained in the indicated number (n) of mice. Grip force (Force) values obtained on the first week were considered 100%. Black symbols, vehicle treated-mice, colored symbols, drug-treated mice. Statistical analysis was conducted using the Mann–Whitney test. $*p < 0.05$. **B.** Spontaneous locomotor (dark phase) activity (left panel) and total running speed (right panel) measured over 20 days in 21-week-old dHT and WT littermates mice treated with vehicle or TMP269+5-Aza. Data points are expressed as mean ($\pm$ S.D; $n = 4-5$ individual mice). $*p < 0.05$ (Mann–Whitney test). The exact *P* value for day 20 is given in the text.

Figure 1A-source data 1: Grip strength in dHT mice is improved after 10 weeks of treatment with TMP269+5-Aza. Each line represents the average grip strength (in grams) per mouse, calculated by averaging 5 measurements obtained from the same mouse. The graphs

show raw data of grip strength before treatment (t=0; grey symbols) and 10 weeks after treatment (red symbols). N=7 for vehicle treated dHT mice and N=12 TMP289+5-Aza treated dHT mice.

Figure 2: The mechanical properties of dHT treated with TMP269+5-aza improve after 15 weeks of treatment. Mechanical properties of EDL and soleus muscle from WT and dHT mice treated with vehicle (WT, n= 8; dHT, n=10) and dHT treated with TMP269+5-Aza, n=13). **A.** Representative traces of twitch and **B.** maximal tetanic force in EDL (150 Hz) muscle from WT and dHT. Force is expressed as Specific Force, mN/mm^2 . **C.** Statistical analysis of force generated after twitch and **D.** tetanic stimulation of isolated EDL muscle. Data points are expressed as Whisker plots (n = 8-13 mice). Each symbol represents the value from a muscle from a single mouse. **E.** Representative traces of twitch and **F.** maximal tetanic force (120 Hz) of soleus muscle from WT and dHT mice. **G.** Whisker plots of force generated after twitch and **H.** tetanic stimulation of isolated soleus muscles. Each symbol represents the value from a muscle from a single mouse (n = 8-13 mice). * $p < 0.05$ ** $p < 0.01$ (ANOVA followed by the Bonferroni post hoc test). The exact P values are given in Supplementary File 2.

Figure 3: Histology of soleus muscles from TMP269+5-Aza and vehicle treated dHT mice. **A.** Analysis of soleus muscles from WT (vehicle treated) and dHT (vehicle and TMP269+5-Aza treated) mice using monoclonal antibodies specific for myosin heavy chain (MyHC) isoforms. Frozen muscle sections were stained with anti-MyHC I antibodies (slow fibers, blue), anti-MyHC IIa antibodies (fast fibers, yellow) and counterstained with anti-laminin antibodies (red). MyHCIIx (fast fibers) are unstained. **B.** Bar plots of fiber type composition of soleus muscles. Left, mean (% , $\pm$ S.E.M.) MyHC I fibers, middle, mean (% , $\pm$ S.E.M.) MyHC IIa fibers, right

mean ($\%$, $\pm$ S.E.M.) MyHC IIx fibers. **C.** Minimal Feret's distribution of type I and type II fibers. Data points are expressed as mean ($\pm$ S.E.M.). For WT vehicle treated a total of 2881 fibers from 3 mice were counted, for dHT vehicle treated a total of 2642 fibers from 3 mice were counted, for dHT TMP269+5-Aza a total of 2983 fibers from 3 mice were counted.

Figure 4: Electrically evoked peak Ca^{2+} transients in muscle fibers from treated dHT compound heterozygous (dHT) mice was rescued by TMP269+5Aza administration. Enzymatically dissociated FDB fibers dissected from 4-6 mice per group, were loaded with Mag-Fluo-4 and electrically stimulated by field stimulation. Black line, vehicle treated WT, grey line, vehicle treated dHT, red line, TMP269+5Aza treated dHT. **A.** Representative Ca^{2+} transient evoked by a single pulse (twitch) of 50 V with a duration of 1 msec. **B.** Whisker plots of peak twitch. Each symbol represents results obtained from a single FDB fiber. **C.** Representative Ca^{2+} transient evoked by tetanic stimulation by a train of pulses delivered at 100 Hz for 300 msec. **D.** Whisker plots of peak transient induced by tetanic stimulation. Each symbol represents results obtained from a single FDB fiber. * $p < 0.05$ (ANOVA followed by the Bonferroni post hoc test). The exact P values are given in Supplementary File 4.

Figure 5: Treatment with TMP269+5Aza reverses RyR1 loss in soleus muscles from dHT mice. **A.** Real time qPCR on RNA isolated from soleus muscles isolated from vehicle treated WT and vehicle treated and TMP269+5-Aza treated dHT mice. Experiments were carried out on muscles isolated from 4 mice per group. RNA isolation and amplification conditions as described in the Methods section. * $p < 0.05$; ** $p < 0.01$ ANOVA followed by the Bonferroni post hoc test. **B.** Western blot analysis of RyR1 content in total homogenates of soleus muscle from WT and dHT

mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 6 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: representative images of blot probed with anti-RyR1 Ab followed by anti-MyHC Ab for normalization. Right panel, data points are expressed as Whisker plots * $p < 0.05$ (ANOVA followed by the Bonferroni post hoc test). C (left panel) representative immunoblots of total homogenates soleus muscles from WT (vehicle) and dHT (vehicle and TMP269+5Aza) mice probed with the indicated antibodies. (Right panel) bar histograms showing the mean $\pm$ S.D. (n=4 mice per group) intensity of the immunopositive band, expressed as % of the intensity of the band in WT (vehicle treated) mice. MyHC was used for loading protein normalization. Forty μ g of protein were loaded per lane and proteins were separated on 7.5% or 10% SDS-PAG. Exact P values are given in the text.

Figure 5B-source data 1: Treatment with TMP269+5Aza reverses RyR1 loss in soleus muscles from dHT mice. Western blot analysis of RyR1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 6 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-RyR1 Ab used in Figure 5B, Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5B-source data 2. Treatment with TMP269+5Aza reverses RyR1 loss in soleus muscles from dHT mice. Western blot analysis of RyR1 content in total homogenates of soleus

muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 6 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-RyR1 Ab used for the quantification analysis shown in the boxplot. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5B-source data 3. Treatment with TMP269+5Aza reverses RyR1 loss in soleus muscles from dHT mice. Western blot analysis of RyR1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 6 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-RyR1 Ab used for the quantification analysis shown in the boxplot. Right panel: images of blot probed with anti-MyHC Ab for normalization.

Figure 5C-source data 1. Treatment with TMP269+5Aza does not affect CSQ1 content in soleus muscles from dHT mice. Western blot analysis of CSQ1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-CSQ1 Ab used for the quantification analysis shown in the bargraph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza;

dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 2. Treatment with TMP269+5Aza does not affect CSQ1 content in soleus muscles from dHT mice. Western blot analysis of CSQ1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-CSQ1 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 3. Treatment with TMP269+5Aza does not affect JP-45 content in soleus muscles from dHT mice. Western blot analysis of JP-45 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 10 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-JP-45 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 4. Treatment with TMP269+5Aza does not affect JP-45 content in soleus muscles from dHT mice. Western blot analysis of JP-45 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 10 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-JP-45 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 5. Treatment with TMP269+5Aza does not affect SERCA1 content in soleus muscles from dHT mice. Western blot analysis of SERCA1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-SERCA1 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 6. Treatment with TMP269+5Aza does not affect SERCA1 content in soleus muscles from dHT mice. Western blot analysis of SERCA1 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-SERCA1 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 7. Treatment with TMP269+5Aza does not affect SERCA2 content in soleus muscles from dHT mice. Western blot analysis of SERCA2 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza. Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-SERCA1 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 5C-source data 8. Treatment with TMP269+5Aza does not affect SERCA2 content in soleus muscles from dHT mice. Western blot analysis of SERCA2 content in total homogenates of soleus muscle from WT and dHT mice treated with vehicle or TMP269+5Aza.

Proteins were separated on a 7.5 % SDS PAGE, blotted overnight onto nitrocellulose. Left panel: images of blot probed with anti-SERCA1 Ab used for the quantification analysis shown in the bar graph. Right panel: images of blot probed with anti-MyHC Ab for normalization. WT Vehicle: Wild type mouse treated with vehicle; dHT Vehicle: dHT mouse treated with vehicle; dHT TMP+Aza; dHT mouse treated with TMP269+5-Aza; unrel sampl: Unrelated Sample (sample belonging to a mouse line of a different project).

Figure 6: Representative EM images of soleus fibres from vehicle treated (A and B) and TMP269+5-Aza treated (C and D) dHT mice. **A.** Representative EM images at low magnification of soleus fibres from vehicle treated and **C.** TMP269+5-Aza treated dHT mice: small arrows point to dilated SR. **B.** and **D.** Higher magnification images showing calcium release units (CRUs, black arrow) and autophagic material (empty arrow, panel B) from vehicle treated and drug treated (D) dHT mice. M = mitochondria. Scale bars: A and C, 1 μ m; B and D, 500 nm.

Appendix 1

The pharmacokinetics and bio-distribution of TMP269, confirm that intraperitoneal injection of 25 mg/kg body weight of TMP269 dissolved in Polyethylenglycol 300 (PEG300) (500 μ l/Kg) and N-Methyl-2-pyrrolidone (NMP) (250 μ L/Kg) results in accumulation of the drug in blood and skeletal muscles. Samples were collected at different time points and the content of TMP269 was quantified by liquid chromatography tandem mass spectrometry (21). The peak blood concentration of TMP269 was achieved approximately 1 hour after injection. Appendix 1-Figure 1A shows that the circulating levels of TMP269 decay within 12 hours. Importantly, the class II-HDAC inhibitor diffuses into skeletal muscle (Appendix 1-Figure 1B), and as expected, its concentration profile in skeletal muscle follows that observed in blood

Figure Legend Appendix 1-Figure 1: Pharmacokinetic profile of TMP269 following intraperitoneal injection in WT mice. A. Concentration-time course of TMP269 in blood. Left panel: mean ($\pm$ S.D., n=4 mice) plasma concentration in mice receiving a single dose of 25 mg/Kg of TMP269. Right panel, mean ($\pm$ S.D., n=4 mice) blood concentration of TMP269 in mice after receiving consecutive doses of 25 mg/Kg of TMP269 at t=0, 24, 48, 72, and 96 hours.

813 Blood samples were taken 10 min before and 1 hour after each intraperitoneal injection. **B.** Mean
814 ($\pm$ S.D.; n=4) TMP269 concentration in skeletal muscle after a single intraperitoneal injection of
815 25 mg/Kg TMP269, during 48 hours.

816

817

Appendix 2

This section confirms the biological effect of daily i.p. injections of TMP269+5-Aza since 165 protein-coding genes were hypomethylation in soleus muscles. Gene Ontology analysis of the hypomethylated genes shows that most of the hypomethylated genes belong to pathways involved in gene transcription, kinase activity and membrane targeting (Appendix 2-figure 1). Furthermore, administration of TMP269+5-Aza increases the acetylation of Lys residues (Appendix 2-figure 2A and Appendix 2-figure 2 source data 1, 2, 3, 4, 5) and of H3K9 (Appendix 2-figure 2B, 2D and Appendix 2-figure source data 1, 2, 3, 4, 5) in total homogenates from FDB fibers isolated from WT and dHT mice, compared to that observed in fibers from vehicle treated WT and dHT mice. This result unequivocally indicates that the inhibition of the deacetylation activity of class IIa HDACs by TMP269 occurs in the nuclei.

Figure Legend Appendix 2- figure 1: Gene Ontology (GO) analysis of hypomethylated genes in soleus muscles from dHT mice treated for 15 weeks with TMP269+5-Aza drug versus vehicle treated dHT mice. Genes showing a ≥ 2.0 -fold decrease in methylation ($P \leq 0.05$) were analyzed by DAVID functional annotation to produce gene clusters (≥ 2 genes/cluster) corresponding to 51 GO annotation terms. GO terms corresponding to biological process (GOTERM_BP_FAT and KEGG_PATHWAY) were extracted and are plotted with the numbers of genes (as a percentage of the total) for each term. GO terms with less than 2% of the total genes were not plotted unless significantly enriched (Benjamini ≤ 0.05).

839

840 **Figure Legend** Appendix 2-figure 2: Daily i.p. injections with TMP269+5-Aza increase

841 **acetylation of Lys residues and of H3K9 in muscles from dHT mice.** WT and dHT mice

842 received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks of

843 treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in cracking

844 buffer (10% glycerol, 5% β -mercaptoethanol, 2.5% SDS, 62.5 mM Tris pH 6.8, 6 M Urea).

845 Protein extracts were loaded on a Tris-Tricine gel, blotted onto nitrocellulose and probed with

846 (A) anti-acetyl-Lys and (B) anti H3K9^{acetyl} antibodies. (C) Shows the immunoreactivity of anti-

847 H3 (histone 3) for loading control. (D) Boxplot analysis of western blots using anti-H3K9^{acetyl}

848 antibodies. The immunoreactivity of the H3K9^{acetyl} positive band obtained in muscles from

849 vehicle-treated WT mice was set to 100%. Each symbol represents the result obtained from a

850 single mouse. *p<0.05 (ANOVA followed by the Bonferroni post hoc test). WT vehicle vs WT

TMP269+5-Aza, P= 0.041; WT vehicle vs dHT vehicle, P=0.037; WT vehicle vs dHT
TMP269+5-Aza, P=0.042; dHT vehicle vs dHT TMP269+5-Aza, P=0.021

**Figure Legend Appendix 2-figure 2 Source data 1. Daily i.p injections with TMP269+5-Aza
increase acetylation of Lys residues and of H3K9 in muscles from dHT mice.** WT and dHT
mice received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks
of treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in
cracking buffer. Protein extracts were loaded onto a Tris-Tricine gel, blotted onto nitrocellulose
and probed as indicated, with anti-acetyl-Lys antibodies, anti H3K9^{acetyl} antibodies and anti- H3
antibodies (loading control). The 3 panels show the immunoreactive bands that were used to
assemble Appendix 2 figure 2. The immunoreactivity of the H3K9^{acetyl} positive band obtained in
muscles from vehicle-treated WT mice was set to 100%.

**Figure Legend Appendix 2-figure 2 Source data 2. Daily i.p injections with TMP269+5-Aza
increase acetylation of Lys residues and of H3K9 in muscles from dHT mice.** WT and dHT
mice received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks
of treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in
cracking buffer. Protein extracts were loaded onto a Tris-Tricine gel, blotted onto nitrocellulose
and probed as indicated, with anti-acetyl-Lys antibodies, anti H3K9^{acetyl} antibodies and anti- H3
antibodies (loading control). The 3 panels show the immunoreactive bands (arrows) that were
used to for the Boxplot analysis of Appendix 2-figure 2. The immunoreactivity of the H3K9^{acetyl}
positive band obtained in muscles from vehicle-treated WT mice was set to 100%.

Figure Legend Appendix 2-figure 2 Source data 3. Daily i.p injections with TMP269+5-Aza increase acetylation of Lys residues and of H3K9 in muscles from dHT mice. WT and dHT mice received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks of treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in cracking buffer. Protein extracts were loaded onto a Tris-Tricine gel, blotted onto nitrocellulose and probed as indicated, with anti-acetyl-Lys antibodies, anti H3K9^{acetyl} antibodies and anti- H3 antibodies (loading control). The 3 panels show the immunoreactive bands (arrows) that were used to for the Boxplot analysis of of Appendix 2-figure 2. The immunoreactivity of the H3K9^{acetyl} positive band obtained in muscles from vehicle-treated WT mice was set to 100%.

Figure Legend Appendix 2-figure 2 Source data 4. Daily i.p injections with TMP269+5-Aza increase acetylation of Lys residues and of H3K9 in muscles from dHT mice. WT and dHT mice received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks of treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in cracking buffer. Protein extracts were loaded onto a Tris-Tricine gel, blotted onto nitrocellulose and probed as indicated, with anti-acetyl-Lys antibodies, anti H3K9^{acetyl} antibodies and anti- H3 antibodies (loading control). The 3 panels show the immunoreactive bands (arrows) that were used to for the Boxplot analysis of of Appendix 2-figure 2. The immunoreactivity of the H3K9^{acetyl} positive band obtained in muscles from vehicle-treated WT mice was set to 100%.

Figure Legend Appendix 2-figure 2 Source data 5. Daily i.p injections with TMP269+5-Aza increase acetylation of Lys residues and of H3K9 in muscles from dHT mice. WT and dHT mice received a daily injection vehicle or 25 mg/kg TMP269+0.05 mg/Kg 5-Aza. After 15 weeks

of treatment, approximately 200 FDBs fibers per mouse were isolated and resuspended in
cracking buffer. Protein extracts were loaded onto a Tris-Tricine gel, blotted onto nitrocellulose
and probed as indicated, with anti-acetyl-Lys antibodies, anti H3K9^{acetyl} antibodies and anti- H3
antibodies (loading control). The 3 panels show the immunoreactive bands (arrows) that were
used to for the Boxplot analysis of of Appendix 2-figure 2. The immunoreactivity of the
H3K9^{acetyl} positive band obtained in muscles from vehicle-treated WT mice was set to 100%.

Appendix 3

The baseline Mag-Fluo-4 fluorescence (in arbitrary units) is similar in FDBs from vehicle treated WT mice (n=91 fibers isolated from n=4 mice), from vehicle treated dHT mice (n=110 fibers isolated from n=6 mice) and from drug treated dHT mice (n=155 fibers isolated from n=5 mice).

Resting fluorescence values in FDB fibers loaded with 10 μ M Mag-Fluo-4. Raw resting fluorescence values (arbitrary units, A.U) were obtained from the light signal originating from a spot of 1 mm diameter of the magnified image of the FDB fiber. The light was converted into an electrical signal by a photomultiplier connected to a Nikon Eclipse TL200-E Fluorescent microscope (Nikon Instruments Inc., Amsterdam, The Netherlands) with a 20x Plan Apo VC Nikon Objective (1.4 NA). Fluorescent signals were acquired using PowerLab Chart7. Each symbol represents the fluorescence value from one FDB fiber.

918 **Supplementary File 1.** List of hypomethylated protein-encoding genes in soleus muscles from
919 dHT mice treated for 16 weeks with TMP269+5-Aza drug versus vehicle treated dHT mice.

920 **Supplementary File 2.** Specific force of EDL and soleus muscle from WT and dHT mice treated
921 with vehicle or TMP269+5-Aza for 16 weeks.

922 **Supplementary File 3.** Fiber type composition of soleus muscles from mice treated for 16
923 weeks with vehicle or TMP269+5-Aza.

924 **Supplementary File 4.** Analysis of electrically evoked calcium transients in single FDB muscle
925 fibers isolated from WT and dHT littermates, treated with vehicle or TMP269+5-Aza (25
926 mg/Kg) for 16 weeks

927 **Supplementary File 5.** Sequence of primers used and targets.

928 **Supplementary File 6.** List of antibodies and suppliers.

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1043 **List of Abbreviations:** 5-Aza, 5-Aza-2-deoxycytidine; CCD, central core disease; CRU,
1044 calcium release units; DHPR, dihydropyridine receptor; DNMT, DNA methyltransferase; ECC,
1045 excitation-contraction coupling; EDL, *extensor digitorum longus*; FDB, *flexor digitorum brevis*;
1046 HDAC, histone de-acetylase; MyHC, myosin heavy chain; MmD, multiminicore disease; SR,
1047 sarcoplasmic reticulum; TT, transverse tubules

1048

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1055 **Data and materials availability:** All data, code, and materials used in the analysis are available

1056 in some form to any researcher for purposes of reproducing or extending the analysis.

1057 There are no restrictions on materials, such as materials transfer agreements (MTAs). All

1058 data are available in the main text or the supplementary materials.

1059