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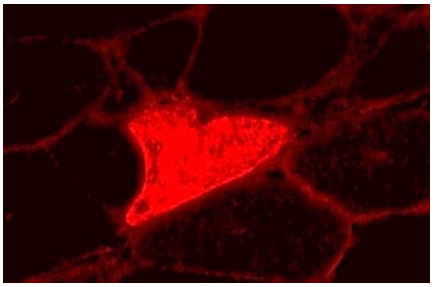
# Unveiling Histone Deacetylase 4 multiple functions in dystrophic skeletal muscle



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## 1. Summary

Histone deacetylase 4 (HDAC4) is a stress-responsive epigenetic factor capable of mediating multiple cellular responses in skeletal muscle, upon different stress conditions. The cytoplasmic functions of HDAC4 in skeletal muscle are poorly characterized so far. HDAC4 expression is upregulated in skeletal muscles of mdx mice, a mouse model for studying Duchenne Muscular Dystrophy (DMD), suggesting a role in this disease. DMD is a genetic, progressive disorder characterized by muscle degeneration and weakness, ultimately leading to the premature death of patients. Pan-HDAC inhibitors are presently in clinical trial for the treatment of DMD, preventing fibrosis and adipogenesis and promoting compensatory regeneration, despite presenting several important limitations. Identifying the specific functions of individual HDAC members is a prerequisite for the development of more selective drugs for the treatment of DMD.

To clarify HDAC4 functions in DMD, we generated dystrophic mice with a skeletal muscle-specific deletion of HDAC4 (mdx;HDAC4mKO mice). The deletion of HDAC4 in skeletal muscle worsens the pathological features of DMD, increasing muscle damage and compromising muscle regeneration and function. HDAC4 affected Fibro Adipogenic Progenitors' potential and decreased their ability to support mdx satellite cells, and compromised mdx satellite cell differentiation via paracrine signals. Moreover, HDAC4 orchestrates membrane repair mechanism in mdx muscles and satellite cells, affecting muscle necrosis, satellite cell survival and myogenic capacity. Importantly, ectopic expression of Trim72, a major player in the membrane repair mechanism, or cytoplasmic HDAC4 rescues mdx;HDAC4mKO mice phenotype *in vitro* and *in vivo*. We demonstrated that HDAC4 cytoplasmic functions are independent of its deacetylase activity and are crucial for allowing a proper response to muscle damage and preserving muscle homeostasis in the DMD context. These findings should be considered for future therapeutic approaches.

## 2. Introduction

### *2.1 Histone Deacetylases (HDACs)*

Histone deacetylases (HDACs) remove the acetyl functional groups from the lysine residues of both histone and non-histone proteins[1]. Histone deacetylation induces the formation of a compacted, transcriptionally repressed chromatin structure, while non-histone protein deacetylation finely tunes their activities and functions[2,3].
To date, 18 different mammalian HDACs have been identified and divided into four classes according to their sequence similarity to yeast counterparts[4,5]: Class I HDACs (HDAC1,2,3 and 8), Class II HDACs (HDAC4,5,6,7,9 and 10), Class III HDACs (Sirtuins), which differ from the others by using $NAD^+$ as a cofactor rather than $Zn^{2+}$, and Class IV HDACs (HDAC11). Moreover, Class II HDACs includes two subgroups: Class IIa (HDAC4,5,7 and 9) and Class IIb HDACs (HDAC6 and 10). Whereas most class I HDACs are ubiquitously expressed and localized in the nuclei, the Class II HDACs are characterized by tissue-specific expression and stimulus-dependent nucleus-cytoplasmic shuttling[6].
The dynamic subcellular localization of Class IIa HDACs depends in part on their $NH_2$-terminal domain, which contains highly conserved serine residues. These serine residues are subjected to signal-dependent phosphorylation in response to different stimuli. Upon phosphorylation, these sites interact with the intercellular chaperone protein 14-3-3, resulting in a conformational change that masks the nuclear localization signal and exposes the conserved nuclear export signal[7,8]. In addition, oxidation of class IIa HDACs results in their exportation from the nucleus[9]. HDAC6, instead, localizes mainly in the cytoplasm and its nuclear import, as well as its tubulin deacetylase activity, is regulated by p300 lysine acetylation[10]. Also HDAC10 can be found in both cytosolic and nuclear fraction; however, how HDAC10 shuttles between the nucleus and cytoplasm is largely unknown[11].

Significantly, all the HDACs are involved in a multitude of biological processes, and their activity impact on human health and disease[12]. Control of cell cycle progression, cell survival and differentiation are among these enzymes' most important roles. Indeed, HDAC deregulation plays a key role in many human diseases, including cancer, neurological diseases, metabolic disorders, inflammatory diseases, cardiac diseases and neuromuscular disorders[6,13–15].

### *2.2 Histone Deacetylase 4 (HDAC4)*

The Human *HDAC4* gene is located on chromosome 2q37.3 and produced 8980 bp mRNA (NM_006037.3) transcript. HDAC4 expression is finely tuned in response to various stimuli, although its transcriptional regulation is still poorly characterized. It has been shown that the transcription factors Sp1 and Sp3 bind to specific consensus sequences on HDAC4 promoter, triggering its transcription[16]. In addition, several miRNAs were characterized to modulate HDAC4 expression: among them, miR-1, miR-29 and miR-140, mir-155, miR-200a, miR-206 and miR-365 acting in different cell types[17–23].

Human HDAC4 protein (1080 amino acids) is characterized by the presence of an N-terminal extension of ~600 residues with distinct regulatory and functional properties[24]. This domain mediates the interactions with transcription factors and co-factors, in addition to containing a nuclear localization sequence[25]. The C-terminal domain of HDAC4, instead, contains a nuclear export signal (NES) and the catalytic domain, which plays a key role in substrate recognition, as well as in the association with the HDAC3·N-CoR transcriptional co-repressor complex[26,27]. HDAC4 protein function, activity, and stability are controlled by post-translational modifications[28,29] (i.e. phosphorylation, carbonylation, sumoylation and ubiquitination). Among them, phosphorylation / dephosphorylation is an essential regulatory modification of HDAC4: phosphorylated S246, S467, and S632 promote the binding

of 14-3-3 chaperone proteins, this interaction allows the HDAC4 translocation from the nucleus to the cytoplasm, resulting in the activation of its target genes. In response to different stimuli, HDAC4 is phosphorylated by calcium/calmodulin-dependent protein kinase (CaMK)[30], Protein kinase D1 (PKD), the extracellular signal-regulated kinases 1 and 2 (ERK1/2)[31], protein kinase A (PKA)[32] and glycogen synthase kinase 3 (GSK3)[33] regulating numerous cellular responses. In the cytoplasm, the phosphatase PP2A interacts with the N-terminal domain of HDAC4 and induces dephosphorylation of the 14-3-3 binding site, thereby promoting the shuttling from the cytoplasm to the nucleus with consequent repression of HDAC target genes[34–36] (Figure 1).

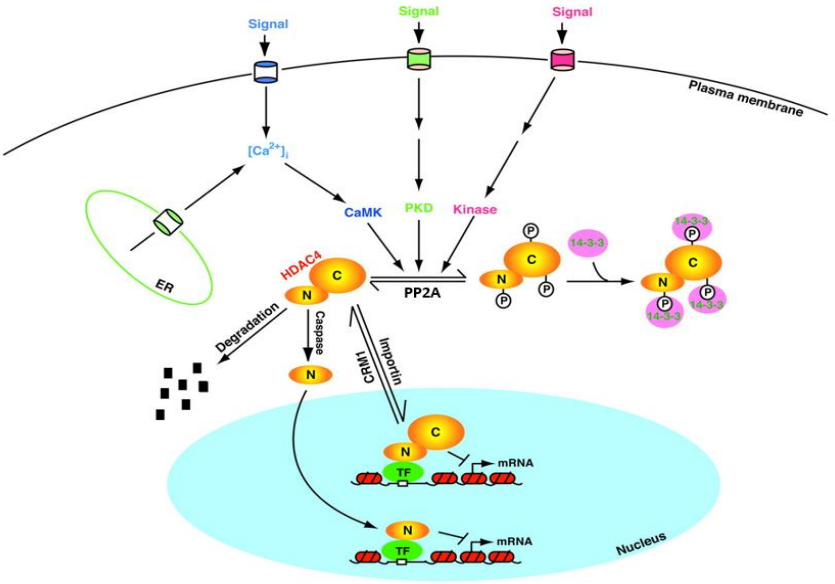



***Figure 1. Mechanisms involved in the regulation of HDAC4 subcellular localization.***

Multiple cell signaling activate several kinases leading to site-specific phosphorylation of HDAC4 and association with 14-3-3 proteins. 14-3-3 binding shifts the trafficking equilibrium of HDAC4 toward cytoplasmic accumulation. PP2A dephosphorylates HDAC4 and dissociate it from 14-3-3 proteins for translocation to the nucleus, where it binds to sequence-specific transcription

factors (TF) to repress transcription. The binding can be direct or mediated by a corepressor. Caspases cleave HDAC4 to generate the N-terminal (N) and C-terminal (C) fragments, with the former translocating to the nucleus as a transcriptional corepressor. HDAC4 may also be subject to proteasomal degradation. (Modified from[36]).

Another HDAC4 modification that modulates its activity is the caspase-dependent processing. HDAC4 is cleaved by caspase-2 and -3 at Asp 289 and sever the carboxy-terminal fragment, which localizes into the cytoplasm, from the amino-terminal fragment, which accumulates into the nucleus. The caspase-cleaved amino-terminal fragment of HDAC4 triggers cell death and acts as a strong repressor of the transcription factor Myocyte Enhancer Factor 2C (MEF2C)[37].

Despite the name, HDAC4, as well as the other class IIa HDAC members, do not properly work on histones, considering also their very low catalytic activity, because of a histidine for tyrosine substitution in their catalytic domain[38]. Several studies have been shown HDAC4 to work in the nucleus as transcriptional co-repressors with NCoR/SMRT complex or to inhibit gene transcription by blocking MEF2 activity[27,39].

HDAC4 plays multiple biological functions, regulating cellular proliferation, growth, and survival. Aberrant HDAC4 expression or activity has been associated with cancer development, such as melanoma[40], breast cancer[41] or Acute Lymphoid Leukemia[42]. The first HDAC4 biological function was described in chondrocytes[43]. Mice lacking HDAC4 die early during the perinatal period due to accelerated chondrocyte hypertrophy, leading to dramatic skeletal abnormalities. The role of HDAC4 in bone development depends on its ability to repress the transcriptional activity of Runt-related transcription factor 2 (Runx2) and MEF2C, two crucial factors for a proper bone formation[43,44].
Several studies highlighted HDAC4 functions in the brain. HDAC4 is highly expressed in this organ, specifically in the neuron

cytoplasm[45]. The brain development is not affected in conditional brain-HDAC4 KO mice, however behavioral abnormalities (i.e. epilepsy) are postnatally manifested[46]. In addition, neurodegeneration of the retina[47] and cerebellum[48] were reported in numerous experimental models in which HDAC4 expression is affected. Coherently, HDAC4 overexpression in a mouse model of retinal degeneration improves neuronal survival through deacetylation of the hypoxia-inducible factor 1α (HIF1α) in the cytoplasm, thus enhancing its activity and avoiding neuronal apoptosis[47]. An additional neuroprotective role of HDAC4, together with HDAC5, was reported following cerebral ischemia injury through inhibition of high-mobility group box 1 (HMGB1), an essential mediator of tissue damage upon acute injury[49]. Altered HDAC4 regulation has been also linked to a number of neurodegenerative disorders, such as Parkinson's disease, and Ataxia-Telangiectasia (AT); in these pathologies nuclear accumulation of HDAC4 in neurons promotes neurodegeneration[50,51].

### *2.2.1 HDAC4 functions in skeletal muscle*

HDAC4 mediates several stress responses in skeletal muscle[15,52–54]. Many of its functions are exercised through the repression of MEF2 activity, an important transcription factor involved in myogenesis and myogenic gene reprogramming.
Considering the early lethality associated with the global deletion of HDAC4, tissue specific knock-out mouse lines were generated to study HDAC4 functions in skeletal muscle. *In vivo* studies have clarified that HDAC4 is dispensable for skeletal muscle development since mice null for HDAC4 in skeletal muscle are available and do not show evident signs of myopathy[55].
As a stress-responsive factor, HDAC4 expression is up-regulated in mouse and human skeletal muscle following denervation, where it regulates muscle atrophy and innervation by mediating several cellular responses[52,56]. Moreover, HDAC4 has been shown to

deacetylate cytosolic proteins, including the MAP3 kinase MEKK2, myosin heavy chain (MHC) isoforms and heat shock cognate 71 kDa protein (Hsc70), and nuclear non-histone protein, as peroxisome proliferator-activated receptor gamma co-activator 1alpha (PGC- 1a), thereby regulating skeletal muscle atrophy and metabolism[57]. Recently, HDAC4 role in mediating nerve-skeletal muscle interaction has been clarified in Amyotrophic Lateral Sclerosis (ALS) context. ALS is a neurodegenerative disease and HDAC4 expression in human skeletal muscle correlates with the severity of the pathology[58]. By using a mouse model of ALS with a tissue specific skeletal muscle deletion of HDAC4, our group highlighted that HDAC4 plays an important role in preserving innervations and skeletal muscle trophism in ALS, likely by modulating the UCP1 gene network[15].

HDAC4 involvement in skeletal muscle adaptation to exercise has been reported in mice and humans. HDAC4, together with HDAC5, are exported from the nucleus during exercise, thereby removing their transcriptional repressive function[59]. In particular, HDAC4/5 dissociation from the GLUT4 promoter enhances GLUT4 transcription in mice upon aerobic exercise, thereby regulating glucose uptake in skeletal muscle[60]. Additional studies reported that HDAC4 plays a role in skeletal muscle metabolic gene regulation[57,61,62]. For instance, HDAC4 mediates SIRT1 repression by IFN-γ in skeletal muscle cells, thereby inhibiting the transcription of genes involved in energy expenditure, such as medium-chain acyl-CoA dehydrogenase, cytochrome-c, cytochrome c-oxydase subunit 4 and carnitine palmitoyltransferase I[63].

Upon injury, HDAC4 expression is up-regulated in skeletal muscle and we demonstrated that it regulates muscle regeneration via autocrine/paracrine signals by releasing soluble factors that influence muscle-stem cell proliferation and differentiation[54]. In addition, by using inducible muscle stem cell-specific KO mouse lines our group, and others, clarified that HDAC4 also regulates satellite cell (SC) proliferation and differentiation potential[64], by repressing the transcription of p21 and Sharp1[53].

HDAC4 expression is also up-regulated in skeletal muscle in several muscular dystrophies, suggesting a role for this protein in these diseases[65,66]. However, the specific HDAC4 functions in muscular dystrophy are yet uncharacterized.

### *2.3. Duchenne muscular dystrophy*

Duchenne Muscular Dystrophy (DMD) is a fatal inherited muscle-wasting disease that affects ~1 in 3500 newborn boys; it is caused by the absence of the structural protein dystrophin because of numerous mutations in the dystrophin gene[67]. Dystrophin, as part of the dystrophin-glycoprotein complex (DGC), anchors the extracellular matrix to the cytoskeleton and has an essential role in stabilizing the sarcolemma during repeated cycles of contraction[68]. The disease onset is between 3 and 5 years, and the disorder progresses rapidly. The absence of dystrophin induces progressive muscle fragility, contraction-induced damage, necrosis, necroptosis, and inflammation. As the disease progresses, myofibers are replaced by fibrotic tissue and fat infiltration, leading to muscle weakness and eventually death, usually in early adulthood[69] (Figure 2).

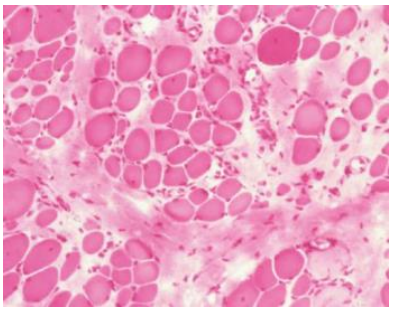


***Figure 2. Histology of DMD muscle***
Skeletal muscle biopsy of DMD patient in advanced stage of disease, stained with hematoxylin and eosin http://medcell.med.yale.edu/histology/muscle_lab/duchenne_muscular_dystrophy.php.

The mdx mouse, which has a point mutation within an exon of the dystrophin gene, is a widely used model for studying DMD[70]. In mdx mice, the dystrophic disease onset occurs between 2 and 3 weeks of age and is characterized by the initiation of muscle necrosis followed by muscle regeneration[71]. Even though in mdx mice muscle dystrophy progression is milder than in DMD patients, likely due to a more efficient regeneration, this mouse model continues to be important for understanding the mechanisms of disease pathogenesis.

### *2.3.1. Degenerative events*

In addition to affects sarcolemma structure and stability, the primary genetic defect in DMD deregulates numerous intracellular signaling, which contribute to the progression of DMD pathogenesis[72]. Indeed, the loss of dystrophin protein affects important mechanisms in skeletal muscle, such as calcium ($Ca^{2+}$) influx[73], infiltration of muscle tissue by inflammatory immune cells[71], the balance between proinflammatory and anti-inflammatory cytokines[72], the activity of proteolytic enzymes[71], oxidative stress[74], autophagy[75], and apoptosis[76]. In addition, ultrastructural alterations and aberrant function of mitochondria have been reported in dystrophic muscles[77,78].

$Ca^{2+}$ plays a key role in skeletal muscle force generation in addition to acts as a secondary transducer that triggers muscle adaptation or degradation[79]. Despite heterogeneous data about intracellular $Ca^{2+}$ concentration in dystrophic muscles have been reported [80–83], numerous studies demonstrate a direct effect of increased cytosolic $Ca^{2+}$ in exacerbating the dystrophic phenotype through several mechanisms[84–86]. For instance, increased cytosolic $Ca^{2+}$ promotes cellular necrosis through calpain activation and mitochondrial permeability transition pore (MPTP) formation[87,88]. Coherently, promoting $Ca^{2+}$ removal from the sarcoplasm through Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA1)

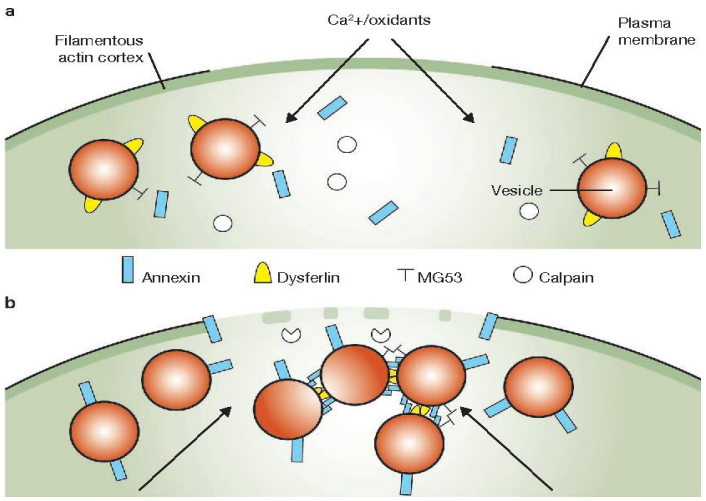
overexpression ameliorates the dystrophic pathology as well as the contraction-induced muscle damage in mdx mice[89,90].
Chronic and consistent inflammation is an important feature of dystrophic muscles. Despite the complexity in inflammatory cell composition in DMD, macrophages represent the primary inflammatory cell type involved in dystrophic muscles. Indeed, a perturbed balance between M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypic populations of macrophages has been reported, thereby affecting the pathology progression in mdx mice[91]. Detrimental effects were reported in mdx mice depleted of macrophages, including decreased SC regenerative potential, suggesting macrophage manipulation as a therapeutic tool[92,93].
A crosstalk between inflammation and Reactive Oxygen Species (ROS) has been proposed[94,95]. Indeed, macrophages and other inflammatory cells, such as neutrophils, are known to be an important source of ROS through the expression of NAD(P)H oxidase (NOX2), a membrane bound complex, catalyzing the conversion of molecular oxygen to reactive O2-. The activity of NOX2 is enhanced in mdx muscles, leading to aberrant $Ca^{2+}$ influx into the myofibers due to a ROS-related overactivation of stretch activated channels (SACs), eventually inducing proteolytic pathways[96]. Moreover, several ROS producing mechanisms were affected in mdx muscle, with a concomitant reduced antioxidant response, thus enhancing oxidative stress and related muscle necrosis through different pathways[74]. For instance, a crosstalk between oxidative stress/lipid peroxidation and NF-κB activation has been reported in mdx mice. As a result, the inflammatory cascades involving tumor necrosis factor alpha (TNF-α), IL-1 β, mitogen-activated protein kinases (MAPKs), cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) is induced, contributing to myofiber death[97].
Another mechanism involved in cell death affecting DMD pathogenesis is the recently identified form of regulated cell death, called necroptosis[98,99]. Necroptosis plays a major role in the inflammation-induced injuries in several tissues, and evidence of

necroptosis in human and mouse dystrophin-deficient muscles have been reported[100]. The TNF superfamily, or other signals that are abundantly present in dystrophic muscles[101,102], trigger a signaling reaction that culminates in the association and auto- and trans-phosphorylation of the receptor-interacting serine/threonine-protein kinases RIPK1 and RIPK3. Once activated, RIPK3 recruits its substrate mixed lineage kinase domain-like (MLKL). Phosphorylated MLKL forms oligomers that translocate to the intracellular membranes and the plasma membrane, which eventually leads to membrane rupture[103,104]. Notably, TNF-α can trigger necroptosis in C2C12 cell line, suggesting that muscle cells can undergo necroptosis upon inflammatory challenge[100]. Morgan et al. demonstrated beneficial effects induced by RIPK3 depletion in mdx mice, in terms of reduced muscle necrosis and fibrosis[100]. However, a recent investigation reports adverse effects of RIPK3 inactivation in dystrophic SC for muscle regeneration and function[105]. On the one hand, these apparently contrasting results confirm the involvement of necroptosis in DMD, on the other hand, underline a not completely understood function of necroptosis in DMD.

### *2.3.2 The sarcolemma repair mechanism*

Several compensatory mechanisms are activated in dystrophic muscles to mitigate myofiber cell death. Enhancing membrane stability and muscle regeneration are the two main responses to counteract muscle degeneration.
Membrane enhanced stability can be achieved in different ways. For instance, a compensatory upregulation of the dystrophin-like protein, utrophin, has been reported in dystrophin-deficient muscle fibers in mdx mice and in some DMD patients[106,107]. Like dystrophin, utrophin interacts with the DGC protein complex to link the cytoskeleton to the extracellular matrix, thereby contributing to sarcolemma stabilization. However, because of functional

differences when compared to dystrophin, utrophin cannot fully compensate dystrophin absence in DMD muscle[106].
Among the strategies to improve muscle membrane stability in DMD, the sarcolemma repair mechanism has been studied by many investigations[108–110]. The membrane repair mechanism is an evolutionary conserved pathway present in many different cell types in which disruptions in the plasma membrane are resealed by the trafficking of intracellular vesicles, able to recognize the injury site and fuse to seal the membrane disruption[111]. Because of the increased susceptibility to myofiber membrane damage in DMD, the sarcolemma repair process is an important compensatory mechanism that allows counteracting muscle necrosis[112]. Dysferlin is the first identified member of the membrane repair machinery in skeletal muscle, and together with MG53 (or Trim72), annexin A1 and caveolin-3 (Cav3) plays a crucial role to orchestrate the membrane resealing process[109,113]. Such process requires several steps and is highly analogous to the patch fusion model of membrane healing proposed for non-muscle cells[114,115] (Figure 3).



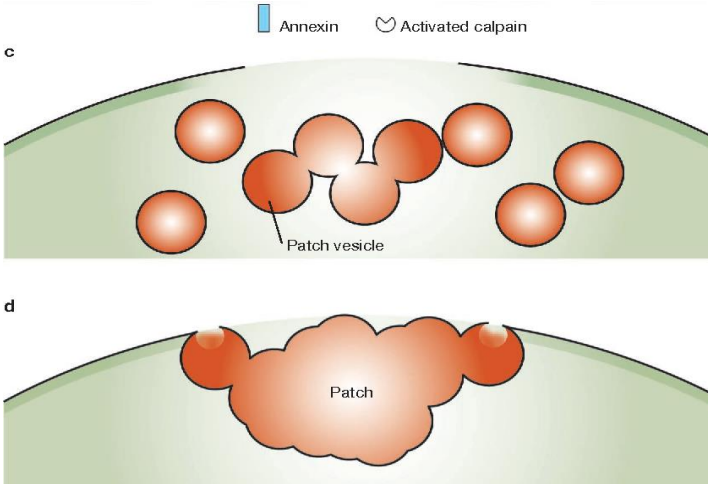



***Figure 3. Model for the membrane repair mechanism***
(**a**) Entry of extracellular calcium and extracellular oxidants triggers a local dynamic response. (**b**) Vesicles transported towards the disruption site by the microtubule motor protein kinesin deliver the required building materials, such as intracellular membrane. The actin-based cortical cytoskeleton disassembles following activation of the protease calpain by calcium, removing a physical barrier to fusion events. (**c**) Vesicles dock and fuse with one another, forming a 'patch vesicle'. Docking is mediated by oxidized MG53. Fusion is mediated by annexin and Dysferlin activated by calcium, and by the SNAREs. (**d**) The patch vesicle fuses with the plasma membrane, completing repair[115].

Under physiological conditions, Dysferlin localizes in the cytoplasm, prevalently closed to t-tubules and to the sarcolemma membrane, where it interacts with annexins A1. Trim72, in its monomeric form, localizes at the intracellular vesicle membrane and the sarcolemma; differently, Cav3 localizes at the caveolae surfaces. A disruption of the sarcolemma membrane results in the reduced intracellular environment's exposure of to the oxidized extracellular milieu, which is sensed by Trim72. Trim72 oligomerizes and triggers the formation of a repair complex, by interacting with Dysferlin, annexins and Cav3. This complex mediates active trafficking of intracellular vesicles to the sarcolemma, where

tethering of the intracellular vesicles to exposed phosphatidylserine of the inner leaflet of the sarcolemma occurs. Eventually, local elevation of $Ca^{2+}$ enables vesicles fusion with the sarcolemma for repair patch formation.
Several studies reported increased expression of the sarcolemma repair proteins in dystrophic muscles, both DMD patients and mdx mice, in which this pathway compensates for the intrinsic damage[112]. Accordingly, enhancing the membrane repair mechanism has been proved to be beneficial in several muscular dystrophies[110]. For instance, ectopic expression of Trim72 protects skeletal muscle from apoptosis and damage in a muscular dystrophy hamster model[116], as well as in mdx mice[117], proposing the administration of recombinant Trim72 as possible treatment for DMD[116]. Coherently, loss of functional dysferlin or Trim72 decrease the membrane repair mechanism, leading to muscular dystrophies and myopathies[113] (i.e. limb-girdle muscular dystrophy type 2B[118], Miyoshi myopathy[118] and a distal anterior compartment myopathy[119]); while a double-deficiency in both dystrophin and dysferlin results in more severe muscular dystrophy in mice[120]. Numerous drugs that enhance sarcolemma repair are currently under investigation to maintain skeletal muscle integrity and to improve muscle functionality in DMD animal models[121], indicating that repairing damaged sarcolemma is a viable approach to reduce dystrophin-deficient myofiber death.

### *2.3.3. Skeletal muscle regeneration*

Skeletal muscle possesses a high capacity to regenerate, and muscle regeneration has been extensively studied since the 19th century. In addition to physiological demand, such as during muscle growth or upon exercise, new muscle fibers are generated in response to muscle damage following injury or muscle degenerative diseases.
Muscle regeneration is a complex biological process involving the coordinated activities of tissue-resident and infiltrating cells, local and systemic signals[122,123]. Upon damage, muscle stem cells, i.e. SC,

are activated and differentiate, promoting maturation of newly formed myofibers. In the early phases of muscle repair, inflammatory cells, such as leukocytes and macrophages, are recruited to the damaged area[124]. These cells amplify the inflammatory response and participate in muscle regeneration, by secreting cytokines and growth factors. Simultaneously, vessels vascularize the newborn myofibers. Besides inflammatory cells, angiogenesis and muscle regeneration are orchestrated by numerous signals, derived from bloodstream and muscle microenvironment[122].
Differently to acute injury, which represents a resolutive self-limiting process, in chronic muscle diseases, such as in DMD, newly generated fibers are also prone to degeneration and muscles undergo constant cycles of fiber degeneration in association with chronic inflammation, eventually leading to the conversion into nonfunctional fibrotic tissue[125]. The effectiveness of muscle regeneration is crucial to counteract DMD progression and to determine the severity of the pathology. Indeed, in DMD patient's regeneration process is efficient until depletion of the SC pool, which occurs early in childhood, while in mdx mice no decline in muscle regeneration was reported for most of the animal life span, resulting in a milder phenotype of the disease.

#### *2.3.3.1 Satellite cells*

SC were first described by Alexander Mauro in 1961 as a population of small mononuclear cells that localized between the plasma membrane of the myofiber and the basement membrane in adult skeletal muscle[126]. A huge number of studies have contributed to defining SC as adult stem cells in skeletal muscle[122,127–129]. Although several types of cells contribute to skeletal muscle regeneration, a key role of SC in this process is now well established[130,131]. During muscle regeneration, SC activation and differentiation recapitulate the embryonic development[132]. Normally quiescent, upon proper stimulation, SC become activated, proliferate, and divide

asymmetrically. One of the daughter cells differentiates in myoblast and then fuse with other myoblasts, or with damaged muscle fibers, to repair the muscle. The other daughter cell maintains a proliferating state or returns to quiescence, ensuring the SC self-renewal[127,133,134]. Defective SC asymmetric division is a hallmark of DMD; indeed, a direct role for dystrophin in regulating SC function has been described[135,136]. Dystrophin protein is highly expressed and polarized in activated SC, where it interacts with the cell polarity regulating kinase MARK2 allowing a polarized distribution of Partitioning Defective Protein 3 (PARD3), needed for a proper mitotic progression of asymmetric SC divisions[135–137]. In the absence of dystrophin-dependent polarity, abnormal mitotic progression occurs, SC undergo cell cycle arrest and may enter in senescence.

Another signaling involved in maintaining SC pool is the Notch pathway. Some studies have demonstrated an interaction between dystrophin and Notch[138,139]; in DMD conditions this interaction is altered thus affecting SC quiescence, proliferation and self-renewal[140]. Moreover, abrogation of asymmetric stem cell division leads to a gradual exhaustion of myogenic progenitors and impaired muscle regeneration[135,136].

In contrast with the SC pool exhaustion hypothesis, several studies have shown an increased number of SC in human and mouse dystrophic muscles[141–143]. However, dystrophic SC display an incomplete maturation in new fibers, which remain small and do not prevent muscle degeneration[144].

It is widely reported that the regenerative capacity of SC decreases with the DMD progression[140], as well as with age[133,145]. This observation has led to the hypothesis of telomere shortening in dystrophic SC, indeed confirmed in mdx mice and DMD children[146,147].

Numerous transcription factors finely tune SC behavior, from their activation to fusion. While quiescent and activated SC do express Pax7, a paired box transcription factor[132,148], once SC are committed to myogenesis, they start expressing other transcription factors, such

as Myf5 or MyoD[149,150]. The transition from myoblast to myotube is mainly regulated by MyoD, which directly regulates the transcription of the other myogenic regulatory factor (MRF) family members, myogenin and MRF4[151]. Also, MEF2 proteins co-operate with MRFs to activate the expression of skeletal muscle terminal differentiation genes, such as MHCs or creatine kinase[152,153]. Myocytes fusion requires the expression of specific genes, including myomaker and myomerger[154,155]. Single-cell analyses by comparing healthy and dystrophic SC have highlighted differential timing in myogenic markers expression[156]. For instance, SC cultures from mdx mice showed an early decline in the expression of MyoD with a concomitant early increase in myogenin expression compared to control cultures, resulting in a more rapid differentiation program in mdx conditions[156].

#### *2.3.3.2 Soluble factors modulating muscle regeneration*

Despite several intrinsic alterations reported in mdx SC compared to healthy ones, numerous evidence supports a key role of dystrophic muscle microenvironment to modulate SC behavior. The pathological muscle environment is deleterious, affecting SC-derived muscle regeneration, as also emphasized in aged mdx mice[122,157–159]. Indeed, SC fate is strictly dependent on a number of soluble factors released by several tissues, including skeletal muscle[123,160]. Blood serum and resident muscle cells are a wide resource of systemic and paracrine factors, including cytokines, hormones (i.e., testosterone) and non-coding RNAs[161–164]. Numerous *in vitro* studies identified different SC responses to single soluble molecules or serum derived from aged or diseased mice[162,165,166]. Several growth factors secreted by cell populations resident within the muscle niche (i.e. FGF, insulin-like growth factors I and II, transforming growth factor β, HGF) have been shown to modulate SC chemotaxis, proliferation and differentiation[167–169]. SC proliferation is supported by mitogens such as FGF and insulin like growth factor (IGF). Elevated FGF level in

the serum of DMD patients was reported[170]. Moreover, a study reported an increased sensitivity to FGF in mdx SC cultured in vitro, compared to SC derived by control mice[156]. IGF-1 it is known to promotes SC proliferation and differentiation, enabling them to fuse to existing muscle fibers[171,172]. Despite significantly greater amounts of IGF-1 were found in plasma and hind limb muscles of mdx mice[173], a concomitant increase in the IGF binding proteins (IGFBPs) levels were detected in DMD patients. The elevated IGFBPs sequester IGF-I, limiting its bioavailability for SC and ultimately resulting in increased skeletal muscle fibrosis[174].
Once activated, SC secrete miRNAs-containing exosomes, which in turn modulate SC behavior[175]. In particular, dystrophic muscle promotes exosomal release containing miR-1, miR-206[176], and miR-133a[177], thereby reducing cell death and muscle degeneration. Inflammatory cells in dystrophic muscles produce several pro-inflammatory cytokines. Among them, TNF-α, which is also produced by muscle cells, plays a central role in the progression of myonecrosis. TNF-α is found to be elevated in DMD and in mdx muscles[178,179], as well as in serum of DMD patients compared to healthy subjects[180]. Coherently, drug therapies designed to inhibit TNF-α or TNF-signaling have been shown to ameliorate muscular dystrophy[181,182]. Others important pro-inflammatory cytokines modulating dystrophic SC fate are interleukin-1 (IL-1) and interleukin-6 (IL-6)[183], whose expression is increased in dystrophic muscles compared to healthy ones. Regarding IL-1, it interferes with the expression of myogenic factors in differentiating myoblasts, by activating the nuclear factor kappa beta (NFkβ)[184]. IL-6 is produced by regenerating myofibers, as well as by several cell types into the muscle niche. Through the JAK1-STAT3 cascade, IL-6 affects SC proliferation and differentiation[185,186].

#### *2.3.3.3 Fibro-adipogenic progenitors*

Fibro-adipogenic progenitors (FAPs) are a quiescent mesenchymal cell population located in the interstitial area of the skeletal muscle[187]. Upon injury, FAPs start to proliferate and contribute to efficient muscle regeneration by positively regulating SC activation and differentiation, through releasing paracrine factors such as follistatin, IL-6, IL-10 and WNT1-inducible-signaling pathway protein 1 (WISP-1), also known as CCN4[187,188].
Under physiological conditions, at the end of the repair process, excessive FAPs return to the quiescent state[187,189,190]. In DMD, as in aging, instead of returning to the quiescent state, FAPs generate fibrous/fatty tissue affecting muscle structure and regeneration[189,191,192]. Thus, FAPs might contribute to DMD pathogenesis by influencing SC activity and by directly promoting fibroadipogenic degeneration.
Numerous studies reported a muscle microenvironment dependent plasticity of FAPs. For instance, both FAP expansion and differentiation have been shown to be mediated by several molecules released by inflammatory cells, such as cytokine IL-4, IL-13, TNF-α and transforming growth factor β1, or released by injured muscles, such as interleukin-15 (IL-15)[189]. During chronic injury, as in DMD, inflammatory cells persist and changes in the cytokine milieu prevents FAP apoptosis, thereby inducing FAP differentiation into persistent matrix-producing cells[193–195]. A recent paper identified Smad2 phosphorylation and the glioma-associated oncogene homolog 1 (GLI1) gene expression as two molecular mechanisms induced by human muscle cell secretome, modulating FAP proliferation and differentiation. Interestingly, these signaling resulted disrupted in aging or in DMD muscle[196]. Notably, when isolated FAPs are cultured *ex vivo* they spontaneously differentiate into adipocytes or fibroblasts, highlighting the crucial role of the muscle microenvironment to inhibit FAP differentiation[194].

### *2.3.4. Therapeutic approaches for the treatment of DMD*

Although no efficient cure is presently available for DMD, different treatments have been developed to restore functional dystrophin (i.e. primary therapies) or to enhance muscle function in a dystrophin independent manner acting on various pathways (i.e. secondary therapies)[197].

The primary therapies include several genetic approaches, such as exon skipping, stop codon readthrough and gene editing, treatment with micro-dystrophins or cell therapies as well. The DMD gene replacement therapy has reached several clinical trials[198]; however, only an exon-skipping orphan drug has been recently approved by the FDA (Food and Drug Administration)[199]. Recently, the new genetic tool based on CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated protein) system gave new hope for the DMD treatment, restoring the dystrophin expression in both germline and postnatal mdx mice or human DMD muscle cells[200,201]. Despite the promising results of these newly developed gene editing strategies, several important limitations need to be considered for future clinical trials. First, the low efficiency of delivery to all affected muscles and heart; second, the high cost for large-scale production of viral particles needed for the numerous patients. Moreover, it is unknown how long the dystrophin expression lasts in edited cells, and if the Cas9 long-term expression in the body leads to side effects, besides considering the risks for off-target mutations in the genome caused by gene editing *in vivo*[201].

Differently from gene therapy, secondary therapies target downstream effects caused by dystrophin mutations to ameliorate life quality and to slow down disease progression[197]. Notably, the lack of dystrophin induces several pathological alterations through multiple pathways, such as inflammation, loss of calcium homeostasis and impaired muscle regeneration. Promoting membrane repair mechanism or muscle regeneration are two of the most desirable goals in order to counteract muscle dystrophy. Despite some of these therapies are effective to delay disease

progression, only combined approaches acting on multiple pathways may be promising to counteract the pathology[197,202].

#### *2.3.4.1. HDAC inhibitors*

HDAC inhibitors (HDACi) are small molecules that inhibit HDAC catalytic activities and restore or increase histone acetylation levels. Clinically relevant HDAC inhibitors are classified by their chemical structure in four classes: the short chain fatty acids (e.g. sodium butyrate, phenylbutyrate, valproic acid or VPA), the epoxyketones (e.g. trapoxin), the benzamide and the hydroxamic acids (e.g. trichostatin A or TSA, suberoylanilide hydroxamic acid or SAHA and givinostat). The latter includes pan-HDACi which not specifically block multiple members of HDAC superfamily through molecular mechanisms yet not fully understood[203,204]. Several studies reveal the involvement of HDACs in mediating muscular dystrophy[205–207], providing a rationale for the use of HDAC inhibitors.

In particular, pan-HDACi promote morphological recovery of dystrophic muscles, favoring muscle regeneration at the expense of fibro-adipogenic degeneration. As reported in[208], HDACi treatment induces an epigenetic reprogramming of FAPs, via an HDAC-myomiR- BAF60 axis driving FAPs toward a pro-myogenic lineage at the expenses of their native fibro-adipogenic phenotype, overall improving regeneration in mdx mice. Moreover, as recently reported, exposure of mdx FAPs to HDAC inhibitors increases the release of extracellular vesicles containing a subset of miRs, which cooperatively target SCs, enhancing regeneration and decreasing fibrosis[209].

A phase II clinical study with the pan-HDACi givinostat showed amelioration of DMD patient muscle histology; though, no functional recovery was reported and any definitive conclusion could be drawn, because of the limited sample size[207]. Moreover, treatment with pan-HDACi resulted not efficient in adult dystrophic mice, and long-term treatment with HDACi, as required for DMD,

has been associated with numerous side effects, raising additional concerns for proposing pan-HDACi as pharmacological therapy[206,210]. Most of these limits may be due to the broad action of the pan-HDACi, considering that different HDAC classes may play different functions and not all them are detrimental in DMD. Delineation of the specific functions of different HDAC classes is a prerequisite for proposing more specific compounds, or combined approaches, that may improve the effectiveness of counteracting DMD.

## 3. Aims

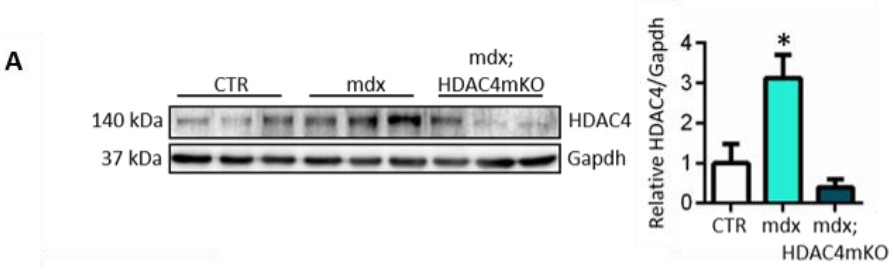
This project aims to unravel Histone Deacetylase 4 (HDAC4) functions in skeletal muscle in Duchenne Muscular Dystrophy (DMD).
HDAC4 is a member of class IIa HDACs that regulates many stress responses in skeletal muscle. HDAC4 expression is upregulated in skeletal muscle of mdx mice, a murine model for studying DMD, suggesting a role in this disease. DMD is a devastating, lethal, genetic disorder characterized by progressive muscle weakness and degeneration. The pan-HDAC inhibitor (HDACi) givinostat showed improvement of DMD histological features in a phase-II study. However, several important limitations are associated with the use of HDACi in DMD. Delineation of the specific functions of different HDAC classes is a prerequisite for proposing more specific compounds or combined approaches, that may improve the effectiveness of counteracting DMD.
The interest of this project is to investigate HDAC4 functions in DMD skeletal muscle, to provide the experimental bases for the development of more effective drugs to be used for the treatment of DMD.

## 4. Results and Discussion

### *4.1 HDAC4 expression and activity are modulated in the skeletal muscle of mdx and mdx;HDAC4mKO mice*

While the function of class I HDACs in DMD has been partially elucidated[211], little is known about the role of class II HDACs. Considering the lethality associated with a global deletion of HDAC4 in mice[43], to investigate the HDAC4 role in DMD by using a genetic approach, mice carrying the skeletal muscle-specific deletion of HDAC4 (Hdac4fl/fl;myogenin-Cre, hereafter referred to as HDAC4mKO), which do not show any obvious phenotype[15], were crossed with mdx mice, obtaining mdx;HDAC4mKO mice. Western blotting (WB) analyses confirmed increased expression of HDAC4 in mdx gastrocnemius (GA) muscles compared to control (CTR) healthy ones and decreased expression in mdx;HDAC4mKO muscles if compared to mdx one, at 6 weeks of age (Figure 1A). The residual expression of HDAC4 in mdx;HDAC4mKO muscles is probably due to the presence of tissues other than skeletal muscle in the protein extract.

To further characterized our experimental model, mdx and mdx;HDAC4mKO satellite cells were isolated and differentiated *in vitro*. HDAC4 expression was evaluated in differentiated myotubes by real-time PCR and WB analyses, confirming a strong decrease in HDAC4 expression in mdx;HDAC4mKO primary cells, compared to mdx ones (Figure 1B).



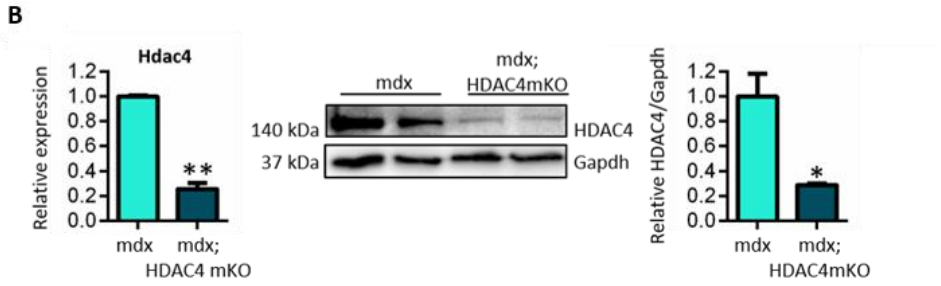



***Figure 1. HDAC4 expression is modulated in mdx and in mdxHDAC4mKO muscle***

**A)** Western blot analyses for HDAC4 in 6 week-old control healthy (CTR), mdx or mdx;HDAC4mKO GA muscles and relative quantification of protein levels. Gapdh was used as loading control. Data are expressed as mean +/− SEM. n=3 mice per condition. One-way ANOVA reveals a significant effect and interaction: *p<0.05 mdx vs CTR and mdx;HDAC4mKO by Tukey's HSD test.

**B)** HDAC4 gene and protein expression by real-time PCR and western blot analyses in mdx and mdx;HDAC4mKO primary myotubes. Data are presented as mean +/− SEM. n=3 mice for each genotype. *p<0.05; **p<0.005 by Student's t test.

Previously published data reported HDAC4 up-regulation in several diseases, such as in Amyotrophic Lateral Sclerosis (ALS)[15], proposing HDAC4 inhibition as a potential treatment. However, our group proved that HDAC4 inhibition upon long-term denervation or in ALS conditions is detrimental for skeletal muscle homeostasis[15,52]. These unpredicted results suggest that HDAC4 may play a key role in the diseases, and its upregulation is needed in skeletal muscle to respond to stress properly.

To study in which cellular compartment HDAC4 mainly localizes in adult mdx muscle, HDAC4 expression was monitored by WB in the nuclear and cytoplasmic fractions, separately. HDAC4 mostly localized in the cytoplasmic fractions of GA mdx muscles (Figure 2).

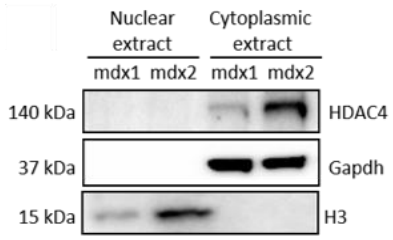



***Figure 2. HDAC4 mainly localized in the cytoplasm of mdx muscles***
Western blot analyses for HDAC4 in nuclear and cytoplasmic extracts of mdx GA muscles at 6 weeks of age. GAPDH and H3 were used as cytoplasmic or nuclear loading control, respectively.

Recent findings are changing the notion that HDAC4 in the cytoplasm is simply an inhibited epigenetic factor, at least in skeletal muscle. For instance, HDAC4 has been demonstrated to deacetylate specific cytosolic substrates, thereby regulating muscle atrophy and metabolism[57] upon denervation. Despite the fact that the lower HDAC4 histone deacetylase activity has been early evinced, few investigations on its possible non-traditional cytoplasmic functions have been reported in skeletal muscle, and none of them clarified HDAC4 functions in dystrophic conditions.

While several studies reported an upregulation of Class I HDACs, which exclusively localize in the nucleus and is associated with deleterious epigenetic modifications in mdx muscle[208,211], little is known about class II HDACs in dystrophic condition.
To exclude any compensatory effects of other HDAC members in our experimental model, we quantified HDAC catalytic activities in mdx and mdx;HDAC4mKO GA muscles, at 6 weeks, using specific fluorogenic substrates containing acetyl-lysine groups 2,3. To verify the specificity of the reactions, the HDACi trichostatin A (TSA) was added in the assay. TSA blunts the class I and class IIb HDAC activities, but not the class IIa, as already reported[212]. The class I, IIa, and I/IIb activities were up-regulated in mdx muscles, compared to healthy ones; HDAC4 deletion in skeletal muscle resulted in a

significant reduction in class IIa activity, as expected, but did not affect class I or I/IIb HDAC activities (Figure 3).

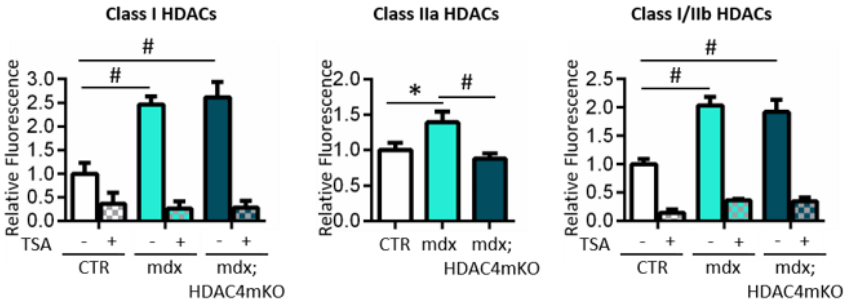



***Figure 3. HDAC4 activity is modulated in mdx and in mdxHDAC4mKO muscle***
HDAC activity assay in mdx and mdx;HDAC4mKO GA muscles at 6 weeks of age. Data are expressed as mean +/− SEM. n=6 mice per condition. One-way ANOVA reveals a significant effect and interaction: *p<0.05; #p<0.01 by Tukey's HSD test.

## 4.2 *HDAC4 deletion in skeletal muscle worsens muscular dystrophy in mdx mice*

To determine if HDAC4 deletion affects the onset and/or the progression of DMD, mdx;HDAC4mKO and age-matched mdx mice were analyzed over time by histological analyses. Muscle dystrophy progression was analyzed in both sexes, by comparing male and female mice independently. Since female mdx;HDAC4mKO mice showed more pronounced differences in respect to mdx littermates, we pursued the analyses in female mice. Muscle morphology and integrity were analyzed by Evans Blue Dye (EBD) staining, over time, in the GA and diaphragm (not shown) muscles, obtaining similar results. mdx;HDAC4mKO GA muscles showed significantly higher EBD+ area as soon as at 3 weeks of age and in all the time points analyzed, except for 6 months, where no gross differences were observed in muscle damage between genotypes (Figure 4A). Higher muscle degeneration was confirmed at 3 and 6 weeks by IgG staining (Figure 4B), as well as by serum

creatine kinase quantification at 6 weeks of age (Figure 4C). This phenomenon is probably due to a more pronounced muscle necroptosis, as proved by elevated levels of the receptor-interacting protein kinase 3 (RIP3/RIPK3), a critical regulator of programmed necroptosis, in mdx;HDAC4mKO mice, compared to mdx littermates (Figure 4D).

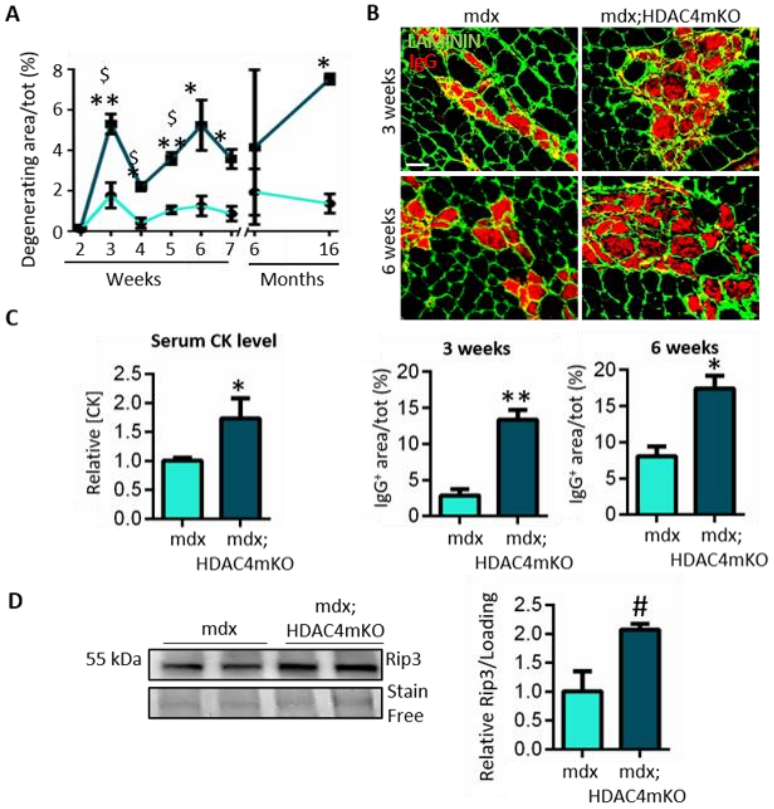
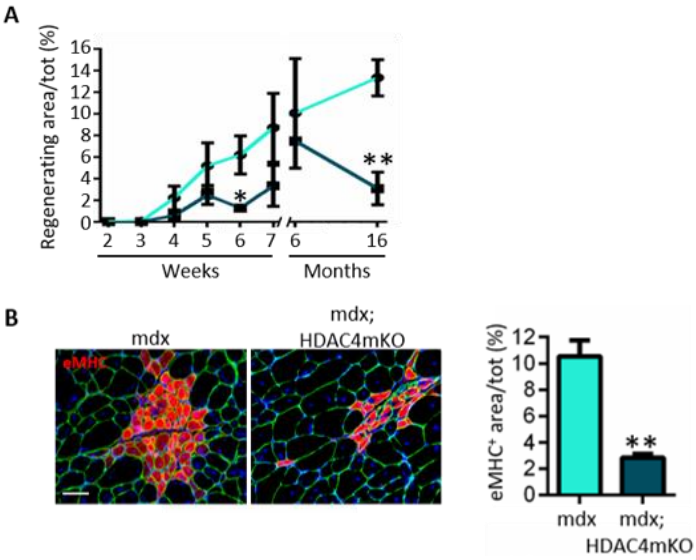



***Figure 4. Deletion of HDAC4 in skeletal muscle exacerbates degeneration in mdx mice***

**A)** Quantification of EBD+ cross-sectional area in GA muscles of mdx and mdx;HDAC4mKO littermates, over time. Data are expressed as mean +/− SEM. n=3 mice per genotype, each time point. One-way ANOVA reveals a significant effect and interaction: *p<0.05 mdx vs mdx;HDAC4mKO; **p<0.005 mdx vs mdx;HDAC4mKO; $ p<0.05 mdxHDAC4mKO vs mdx;HDAC4mKO of the previous time point by Tukey's HSD test. **B)** Representative images and

quantification of IgG+ cross sectional area (CSA) in mdx and mdx;HDAC4mKO GA muscles at 3 and 6 weeks of age. Data are expressed as mean +/− SEM. n=3 mice per genotype, each time point. *p<0.05; **p<0.005 by Student's t test. **C)** Creatine kinase levels in sera of mdx and mdx;HDAC4mKO mice at 6 weeks of age. Data are expressed as mean +/− SEM. n=7 mice per genotype. *p<0.05 by Student's t test. **D)** Representative western blot and densitometric analyses of Rip3 in mdx and mdx;HDAC4mKO GA muscles, at 6 weeks of age. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over mdx mice. n=3 mice for each genotype. #p<0.01 by Student's t test.

Muscle regeneration was analyzed by hematoxylin and eosin (H&E), and quantified by counting regenerating, centronucleated fibers in GA cross-sections over time. mdx;HDAC4mKO GA muscles showed a significantly lower regenerating area in respect to mdx littermates, starting from 4 weeks of age and in all the time points analyzed, with significant differences at 6 weeks and 16 months (Figure 5A). Reduced muscle regeneration was confirmed by embryonic myosin heavy chain (eMHC) immunostaining (Figure 5B), and further proved by significantly reduced myogenin protein levels by WB analyses in mdx;HDAC4mKO GA muscles compared to mdx littermates, at 6 weeks (Figure 5C).



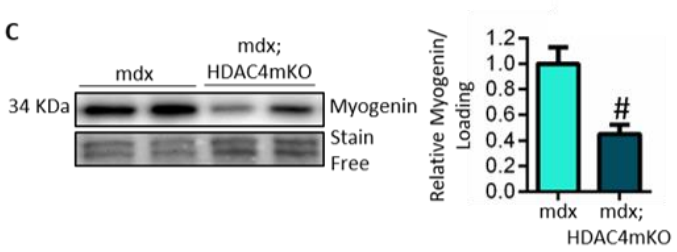



***Figure 5. Deletion of HDAC4 in skeletal muscle impairs regeneration in mdx mice***

**A)** Quantification of centronucleated regenerating myofiber cross-sectional area in GA muscles of mdx and mdx;HDAC4mKO littermates, over time. Data are expressed as mean +/− SEM. n=3 mice per genotype, each time point. One-way ANOVA reveals a significant effect and interaction: *p<0.05 mdx vs mdx;HDAC4mKO; **p<0.005 mdx vs mdx;HDAC4mKO by Tukey's HSD test.
**B)** Representative pictures of IF for eMHC (red) in GA muscles of mdx and mdx;HDAC4mKO mice at 6 weeks of age and quantification of the eMHC+ CSA. Data are expressed as mean +/− SEM. n=3 mice per genotype. **p<0.005 by Student's t test.
**C)** Representative western blot and densitometric analyses of myogenin in GA muscles of 6 week-old mdx and mdx;HDAC4mKO mice. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over mdx mice. n=4/5 mice for each genotype. #p<0.01 by Student's t test.

We then verified whether the histological and molecular differences between the genotypes affect muscle performance. Muscle functionality was evaluated at 6 weeks and 6 months by subjecting mice to a downhill treadmill exercise protocol[213] and at 16 months, by using a less stressful exercise protocol[214]. Running time and distance to exhaustion were measured.
Importantly, mdx;HDAC4mKO mice were significantly less performing at all the time points compared to mdx littermates (Figure 6), indicating a global and severe muscle dysfunction.

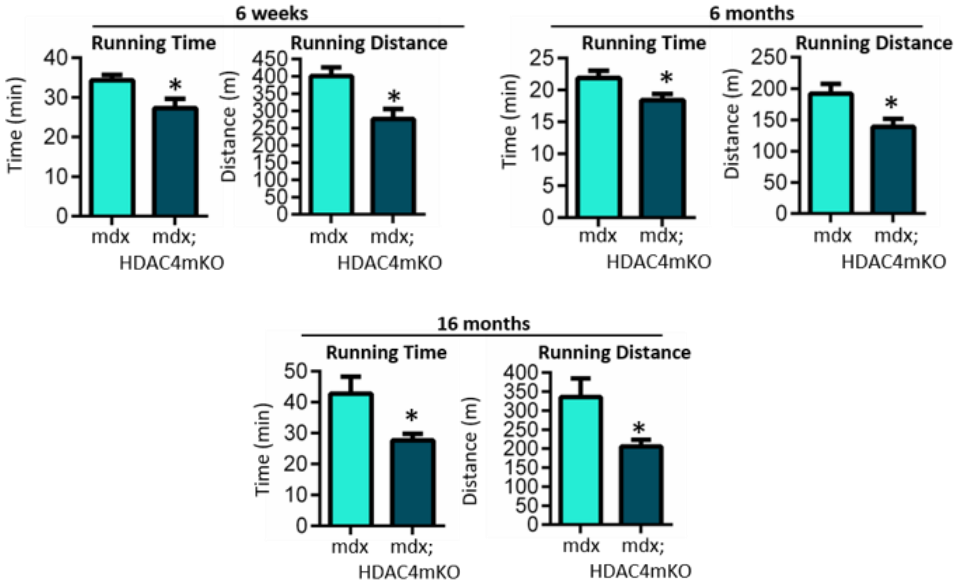



***Figure 6. Deletion of HDAC4 in skeletal muscle affects muscle functionality in mdx mice***

*Muscle performance of mdx and mdx;HDAC4mKO mice by treadmill test, at 6 weeks, 6 and 16 months. Data are presented as mean +/− SEM. n=6 mice per genotype. *p<0.05 by Student's t test.*

### *4.3 HDAC4 affects satellite cell potential and survival in mdx mice*

Based on impaired skeletal muscle regeneration in mdx;HDAC4 mKO mice, we wondered whether HDAC4 affected SC potential in mdx mice. SCs were isolated and induced to differentiate; terminal differentiation was assessed by immunofluorescence (IF) for Myosin Heavy Chain (MHC). SCs isolated from mdx;HDAC4mKO mice showed significantly reduced differentiation and fusion abilities, as quantified by the differentiation (i.e. the number of MHC+ myonuclei over the total nuclei) and fusion (i.e. the number of myonuclei in myotubes over the total nuclei) indexes (Figure 7A). Moreover, a significant reduction in the mdx;HDAC4mKO SC number was quantified, in respect to mdx SCs (Figure 7A).

Decreased SC differentiation in mdx;HDAC4mKO cells was also corroborated by analyzing the gene expression of two myogenic markers, i.e. embryonic MHC (eMHC) and muscle creatine kinase (MCK), by real-time PCR (Figure 7B).

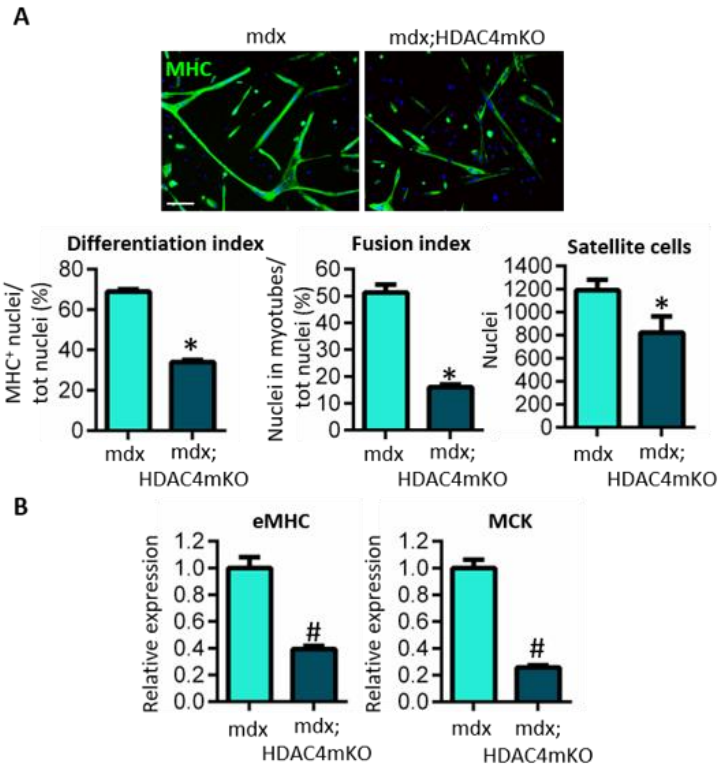



***Figure 7. Deletion of HDAC4 in skeletal muscle affects SC differentiation in mdx mice***

**A)** Representative images of IF for MHC in mdx and mdx;HDAC4mKO satellite cells, after three days in differentiation medium. Scale bar: 100 μm. Quantification of differentiation and fusion indexes and number of satellite cells. Data are presented as mean ± SEM. n=4 mice per genotype. *p<0.05 mdx vs mdx;HDAC4mKO by Student's t test. **B)** Expression levels of myogenic markers in mdx;HDAC4mKO satellite cells, compared to mdx, by real-time PCR. Data are presented as mean ± SEM. n=4 mice per genotype. #p<0.01 by Student's t-test.

Since a reduction in mdx;HDAC4mKO SC number was observed, SC death was analyzed by Tunel assay in differentiating myotubes: mdx;HDAC4mKO SCs showed a significant increase in Tunel+ nuclei, compared to mdx SCs, paralleled to a decreased in the overall number of SCs (Figure 8A). Being necroptosis one of the mechanisms involved in cell death in DMD[100], Rip3 levels were quantified in differentiating SCs. mdx;HDAC4mKO SCs showed higher Rip3 levels in respect to mdx SCs (Figure 8B), indicative of higher necroptosis.

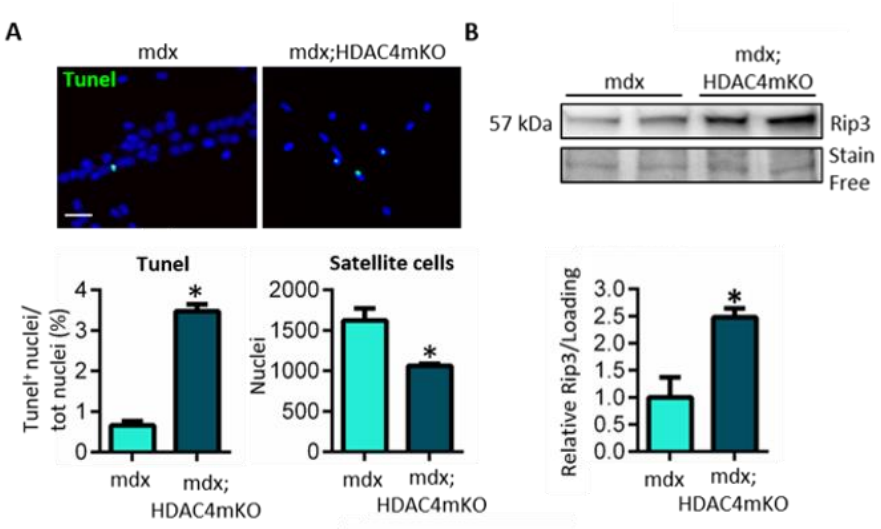



***Figure 8. mdx;HDAC4mKO SC are more susceptible to cell death***
**A)** Representative images of Tunel assay in mdx and mdx;HDAC4mKO satellite cells after two days of differentiation. Scale bar: 100 μm. Quantification of the Tunel+ cells and number of satellite cells. Data are presented as mean ± SEM. n=4 mice per genotype. *p<0.05; mdx vs mdx;HDAC4mKO by Student's t test.
**B)** Representative western blot and densitometric analyses of Rip3 protein levels in mdx and mdx;HDAC4mKO satellite cells. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over mdx mice. n=3 mice for each genotype. *p<0.05 by Student's t test.

Despite the deficit in SC differentiation, mdx;HDAC4mKO mice properly developed skeletal muscle (Figure 9A), without apparent deficit in muscle CSA or fiber CSA distribution at 2 weeks of age (Figure 9B).

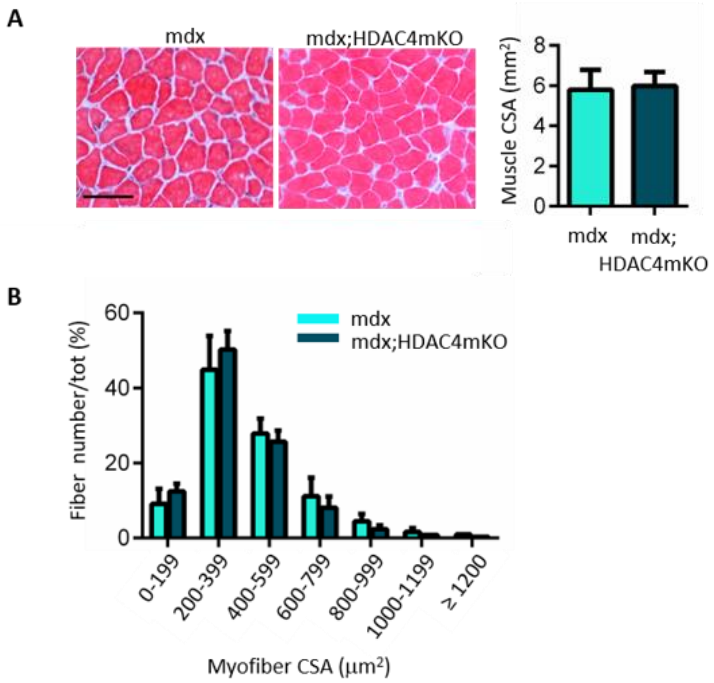



***Figure 9. Deletion of HDAC4 in mdx mice does not affect muscle development*** ***A)*** *Representative images of GA muscle histology at 2 weeks of age. Scale bar: 50 µm.* ***B)*** *GA muscle CSA and myofiber CSA distribution of mdx and mdx HDAC4mKO GA muscles at 2 weeks of age. Data are expressed as mean +/− SEM. n=3 mice per each genotype.*

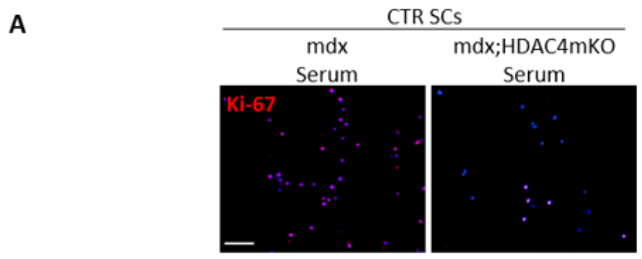
*In vitro* studies with myogenic precursor cell lines reported that the HDAC4 cytoplasmic translocation, and thus MEF2 derepression, is necessary for a correct myoblast differentiation[39,66]. HDAC4 deletion in differentiating skeletal muscle cells *per se* does not affect the myogenic potential of muscle precursor cells[54], differently from

mdx;HDAC4mKO SCs, indicating a specific HDAC4 function in the absence of dystrophin.

These results indicate that HDAC4 deletion in dystrophic skeletal muscle correlates with higher muscle damage, accompanied with reduced muscle regeneration, increased SC death and impaired SC ability to differentiate.

*4.4 HDAC4 paracrine functions in DMD*

Our previous data showed HDAC4 involvement in the release of muscle-derived soluble factors affecting muscle regeneration following acute injury[54]. Based on these results, we speculated that HDAC4 mediates the release of soluble factors also in the dystrophic context, affecting SC differentiation. To test this hypothesis, we cultured control SCs with conditioned medium by using either mdx;HDAC4mKO or mdx mouse serum. SC proliferation was assessed in growing conditions by IF for Ki-67, while terminal differentiation was assessed by IF for MHC. mdx;HDAC4mKO serum significantly reduced the proliferating Ki-$67^{+}$ SC number, and the total SC number, if compared with mdx sera (Figure 10 A). Moreover, mdx;HDAC4mKO serum compromised SC differentiation and fusion, in addition to reduce SC number (Figure 10 B).



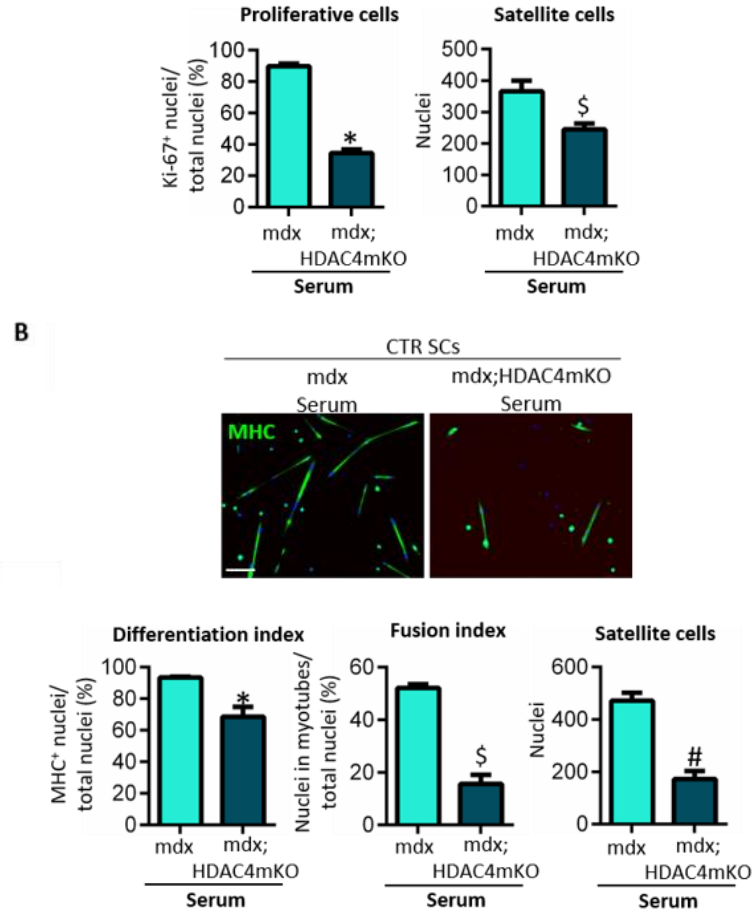



***Figure 10. HDAC4 from skeletal muscle regulates circulating factors to affect SC proliferation, differentiation, and fusion in DMD.***
**A)** Representative images of IF for Ki-67 in CTR SCs after 24 hours in culture with mdx or mdx;HDAC4mKO serum conditioned media. Scale bar: 100 µm. Quantification of the proliferating Ki-67+ SCs, over the total SCs, and of SC nuclei. n=3 mice per genotype. *p<0.05; $p<0.001 by Student's t test. **B)** Representative images of IF for MHC in CTR SCs cultured with mdx or mdx;HDAC4mKO serum conditioned media. Scale bar: 100 µm. Quantification of differentiation and fusion indexes and number of SCs. Data are presented as mean ± SEM. n=3 mice per genotype. *p<0.05; #p<0.01; $p<0.001 by Student's t test.

These results indicate that HDAC4 in DMD muscles mediates the production and/or the release of circulating factors able to affects SC proliferation and differentiation.

Since inflammatory cells may affect SC biology in regenerating or dystrophic muscles[92,124], the inflammatory composition was evaluated in mdx and mdx;HDAC4mKO muscles by flow cytometry analysis. Importantly, no significant inflammatory cell composition changes were quantified in mdx;HDAC4mKO mice, compared to mdx one (Figure 11), thus supporting the muscle as a direct source of soluble factors affecting SC biology in mdx;HDAC4mKO mice.

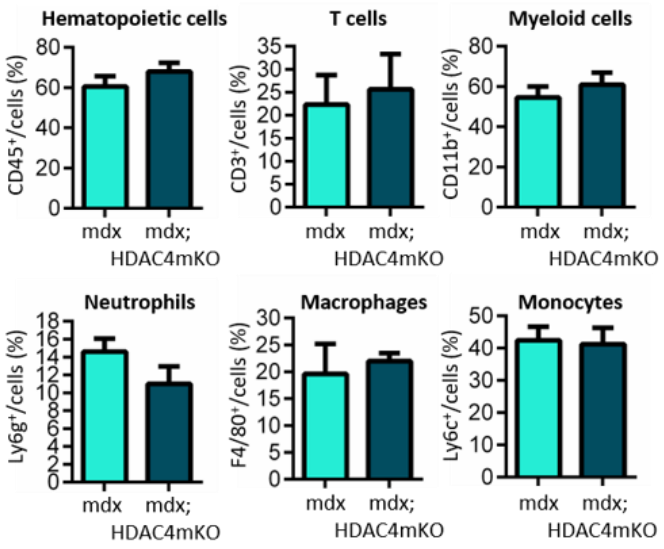



***Figure 11. HDAC4 does not affect inflammatory cell composition in mdx muscle***
Quantification of immune cell composition in mdx;HDAC4mKO and mdx muscle derived cells by flow cytometry analysis. n=6 mice per each genotype.

We further characterized our experimental model by analysing FAPs, which are known to mediate the beneficial effects of HDACi

in mdx mice[208]. Indeed, in young mdx mice, HDACi inhibit FAP adipogenic potential, while enhancing their ability to promote differentiation of adjacent SC, through up-regulating and promoting the release of soluble factors [195,209].

On these bases, we verified whether HDAC4 from skeletal muscle influences FAPs potential in our experimental model. FAPs were isolated from young mdx;HDAC4mKO and mdx mice, cultured and induced to adipogenic differentiation, as described in[206]. FAPs were stained with Oil red O (ORO) to mark neutral triglycerides and lipids, adipogenic potential was quantified by measuring the $ORO^+$ area. Strikingly, mdx;HDAC4mKO FAPs showed higher adipogenic potential with respect to mdx FAPs (Figure 12).

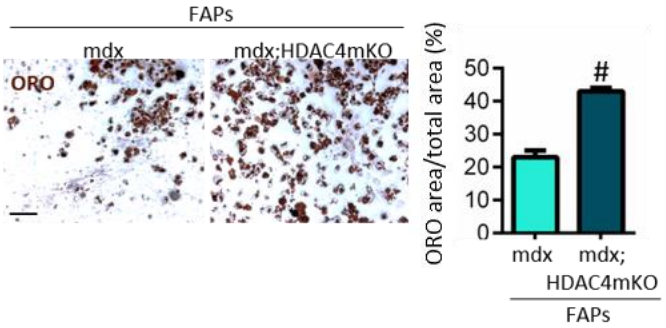



***Figure 12. Deletion of HDAC4 in mdx skeletal muscles affects FAPs potential***
Representative images of mdx and mdx;HDAC4mKO FAPs cultured in adipogenic induction medium and stained with ORO. Scale bar: 50 μm. Quantification of adipogenic potential. Data are expressed as mean +/− SEM. n=3 mice per each genotype; #p<0.01 by Student's t test.

FAPs are the mainly source of fat deposition in aged mdx muscles[215] therefore we wondered whether mdx;HDAC4mKO muscles show increased adipogenic infiltration in vivo. Fat deposition was analyzed by ORO staining in 16 month–old mdx;HDAC4mKO and mdx GA muscles. A significant increase of adipogenic content in

mdx;HDAC4mKO GA muscles compared to mdx ones was quantified (Fig. 13).

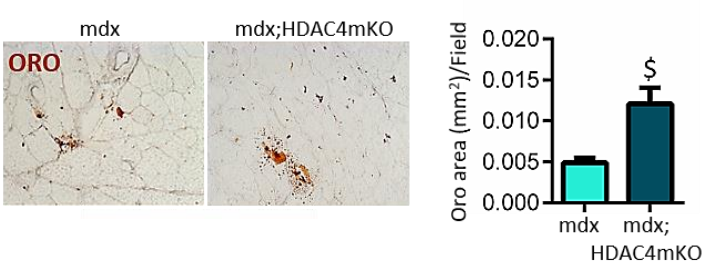



***Figure 13. Deletion of HDAC4 in mdx skeletal muscles exacerbates fat deposition***
Representative images of mdx and mdx;HDAC4mKO GA muscles at 16 months stained with ORO and quantification of fat accumulation in muscles. Data are expressed as mean +/− SEM. n=3 mice per each genotype; $p<0.001 by Student's t test.

The pro-adipogenic commitment of FAPs is influenced by several pathways in dystrophic muscles. Inflammatory environment and myofiber-released signals cooperate to inhibit FAPs adipogenesis in young mdx muscle avoiding fat deposition. Differently, decreased inflammation in old mdx mice remove important signals to control FAPs behavior, which strictly depends by muscle signaling at this stage[194]. Our result indicate that fat deposition is exacerbated in mdx;HDAC4mKO mice compared to mdx ones, thus confirming HDAC4 signaling crucial to modulate FAPs behavior in dystrophic context.

Considering the FAP crucial role in mediating SC behavior[195,209], we wondered whether HDAC4 influences FAPs ability to modulate mdx SC biology.
We co-cultured FAPs and mdx SCs using the cell-insert system, which provides a two-compartment culture system and allows exchange of media, nutrients, and molecules between two different cell populations. mdx SCs were plated in the bottom of the plate,

while either mdx;HDAC4mKO or mdx FAPs cells were plated on the upper insert. Transwell co-cultures were maintained in GM for 3 days, followed by further 3 days in myogenic induction medium and then harvested for analyses. Strikingly, mdx;HDAC4mKO FAPs inhibited mdx SC differentiation, as shown by IF for MHC and quantified by the differentiation and fusion indexes, compared to mdx SCs cultured with mdx FAPs (Figure 14). In addition, mdx;HDAC4mKO FAPs induced a reduction in mdx SCs nuclei, as quantified in Figure 14.

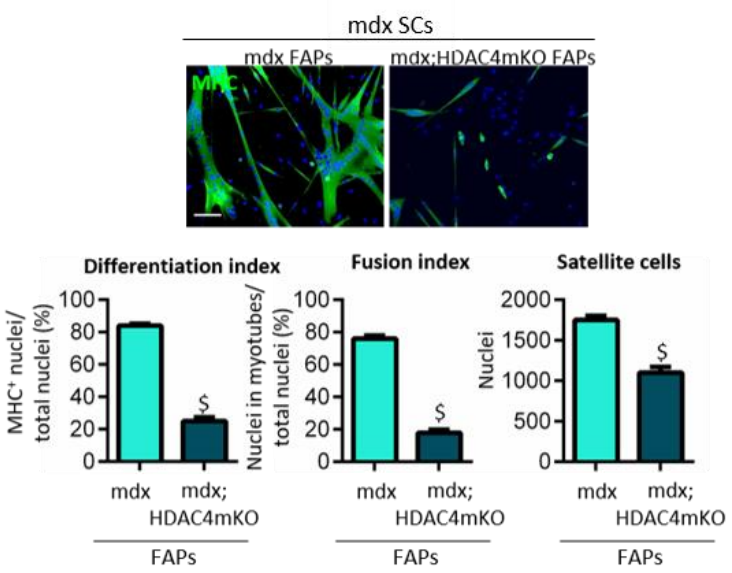



***Figure 14. mdx;HDAC4mKO FAPs affect mdx SC biology***
Representative IF images for MHC of mdx SCs co-cultured with either mdx or mdx;HDAC4mKO FAPs. Scale bar: 50 μm. Quantification of differentiation and fusion indexes and SC number. Data are presented as mean +/− SEM. n=3 mice per genotype; $p<0.001 by Student's t test.

To prove that HDAC4 from skeletal muscle affects FAPs via soluble factors, mdx and mdx;HDAC4mKO single myofibers were isolated from 3 week-old mice and cultured *in vitro* as in[216,217]. Three days

later, conditioned media (CM) were collected, centrifuged, filtered and stored at 4°C, as described in[218]. CM were used to culture either mdx SCs or FAPs.
mdx SCs were cultured for 3 days with either mdx or mdx;HDAC4mKO myofiber CM, then CM was replaced with a conditioned differentiation medium (DM), consisting of a 1:10 dilution of CM. Interestingly, no significant differences in terms of mdx SC differentiation or survival were detected between cells cultured with mdx or mdx;HDAC4mKO myofiber CM (data not shown).
FAPs were cultured for 7 days with either mdx or mdx;HDAC4mKO myofiber CM and then exposed for 3 days to adipogenic induction medium. After additional 3 days of adipogenic maintenance media, FAPs were stained with ORO, to verify their adipogenic potential (Figure 15). mdx;HDAC4mKO myofiber CM was sufficient to affect mdx FAPs, which resulted more pro-adipogenic than mdx FAPs cultured with mdx myofiber CM.

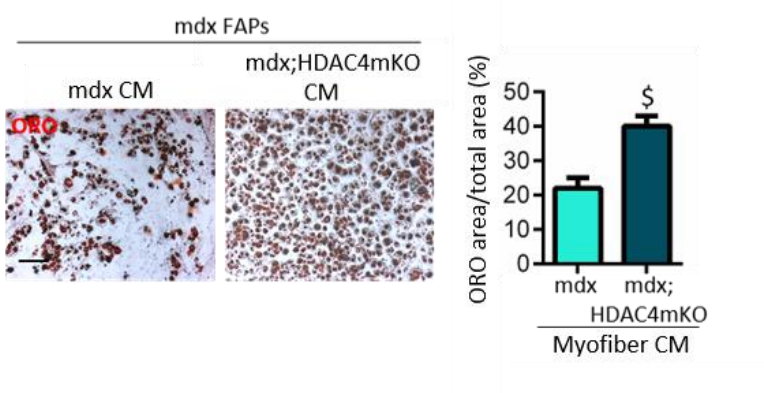



***Figure 15. mdx;HDAC4mKO fiber conditioned media affects FAP biology***
Representative pictures of mdx FAPs cultured with conditioned media (CM) from either mdx or mdx;HDAC4mKO myofibers and stained with ORO. Scale bar: 50 μm. Quantification of adipogenic potential. Data are presented as mean +/− SEM. n=3 mice per genotype; $p<0.001 by Student's t test.

These data indicate that HDAC4 from mdx skeletal muscle mediates soluble factors that affect mdx FAPs, highly prone to differentiate

into adipocytes. Moreover, mdx;HDAC4mKO FAPs humper mdx SC differentiation, revealing another crucial role of HDAC4 in mediating the release of soluble factors from mdx muscle affecting the functional interaction between FAPs and SCs.

Overall, we speculate that HDAC4 plays multiple functions in dystrophic muscles, by affecting SCs in cell-autonomous manner and by releasing soluble factors that influence both mdx FAP and SC behavior. In addition to regulate mdx;HDAC4mKO SC differentiation and survival, HDAC4 regulates the release of soluble factors from myofibers able to affects FAPs biology, as shown by conditioned-medium experiments *in vitro*. Moreover, inflammatory cell composition seems to be unaffected in mdx;HDAC4mKO mice, excluding a possible secondary effect on FAPs due to a widespread muscle degeneration. Myofibers are source of myokines and soluble molecules able to affect several cell types, including FAPs. For instance, upon muscle injury, myofibers produced IL-15 able to inhibit adipogenesis of FAPs[219]. In addition, skeletal muscle is known to produce extracellular vesicles (EVs) in response to different stimuli (i.e. exercise and pathologies), thereby affecting muscle environment [220,221]. Our results indicate HDAC4 involvement to modulate soluble factors or EVs content from myofibers which in turn affect FAPs behavior in mdx mice. Importantly, in vitro conditioned-medium experiments demonstrated that HDAC4 from skeletal muscle does not directly influence mdx SCs behavior but needs FAPs as cellular mediators for exerting its function. Further on going- experiments are aimed to characterize mdx and mdx;HDAC4mKO myofiber-secretome, in order to identify the soluble factors able to affect FAPs biology.

Recently, an important role for HDACs in affecting FAP-derived EV content in DMD has been reported[209]. Exposure of mdx FAPs to pan-HDACi increases the expression and the release of miR-206 in EVs, favoring muscle regeneration and reducing fibrosis and inflammation in mdx mice. Our results demonstrate a crucial role

for HDAC4 in skeletal muscle: if deleted, mdx FAPs showed a more pro-adipogenic phenotype and decreased ability to promote mdx SC differentiation. Our results are important considering that pan-HDACi are systemically administrated in DMD patients. In the future experiments, we plan to analyze FAP EV content in our experimental model.

Despite our data are in apparent contrast with the role of HDACi in dystrophic muscles, some important differences mark our experimental model from treating mdx mice or FAPs with HDACi: (1) pan-HDACi unspecifically inhibit all members of HDAC superfamily, differently from having a genetic deletion of a single member of class IIa HDACs; (2) systemic administration of HDACi affects all tissues, differently from studying the effects on a tissue-specific KO mouse; (3) administration of HDACi starting from 1,5 months may provide different results from harboring HDAC4 deletion since the embryonic stage E8.5, as in mdx;HDAC4mKO mice.
Nevertheless, our results show that HDAC4 from skeletal muscle influences FAPs, which in turn affects mdx SC biology, thus proving a protective function of HDAC4 that needs to be preserved in DMD conditions. Further studies are needed to clarify which are the soluble factors regulated by HDAC4 in mdx muscles and if HDAC4 function is altered by HDACi treatment in mdx mice, in order to further improve the use of HDACi as therapeutic approach for the treatment of DMD.

*4.5 HDAC4 affects membrane stability and repair in mdx mice*

With the aim to characterize the molecular mechanisms that HDAC4 mediates in DMD, we focused our attention on the enhanced muscle degeneration observed and quantified in mdx;HDAC4mKO muscles. First, to prove that HDAC4 affects muscle fragility in mdx muscles, muscle damage was induced by a downhill treadmill exercise at 6 months of age, when no significant differences were observed between genotypes in terms of muscle degeneration. Quantification of EBD+ area revealed that exercise induced a significant increase in muscle necrosis; however, a higher increase was found in exercised mdx;HDAC4mKO GA muscles compared to exercised mdx ones (Figure 16).

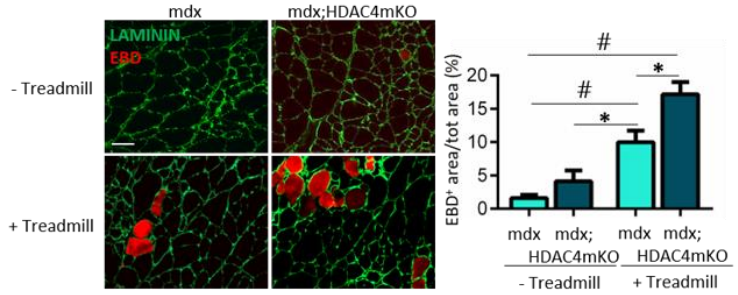



***Figure 16. HDAC4 affects membrane stability in mdx mice***
Representative images of mdx and mdx;HDAC4mKO GA muscle at 6 months of age with or without treadmill exercise, labelled with EBD (red) and laminin (green) and quantification of EBD+ cross sectional area. Scale bar: 50 μm. Data are expressed as mean +/− SEM. n=5 mice per genotype. One-way ANOVA reveals a significant effect and interaction: *p<0.05; #p<0.01 by Tukey's HSD post hoc test.

Of note, HDAC4 deletion in skeletal muscle *per se* does not affect muscle fragility or performance. Indeed, HDAC4mKO mice showed no sign of necrosis in GA muscles (Figure 17A), or differences in muscle performance (Figure 17B) upon the downhill

treadmill exercise protocol, when compared to CTR healthy mice, indicating a specific HDAC4 function in DMD context.

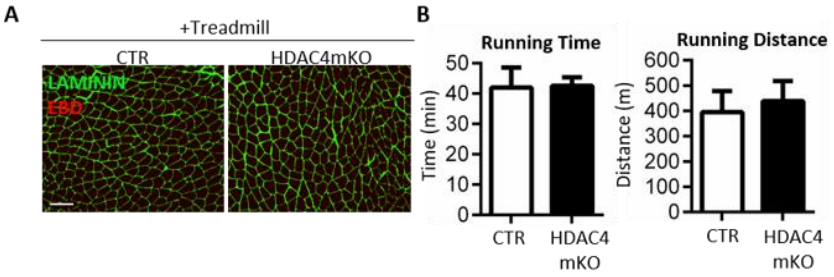



***Figure 17. Deletion of HDAC4 in skeletal muscle of healthy mice does not affect muscle homeostasis or performance***
**A)** Representative images of control healthy (CTR) and HDAC4mKO GA muscles at 6 months of age, labelled with EBD (red) and laminin (green), after a downhill exercise protocol to exhaustion. Scale bar: 100 um. **B)** Muscle performance of CTR and HDAC4mKO mice by treadmill test, at 6 months. Data are presented as mean +/− SEM. n=3-5 mice per genotype.

In the absence of dystrophin, sarcolemma is more fragile and thus more prone to contraction-induced damage[68,222]. HDAC4 deletion sensitizes mice to dystrophic abnormalities, enhancing muscle fragility upon treadmill exercise, compared to mdx one.
One of the principal mechanisms triggered by sarcolemma damage is the membrane repair mechanism, which is normally upregulated in dystrophic conditions, acting to counteract muscle fiber death. Considering the higher muscle fragility observed in mdx;HDAC4mKO muscles, we investigated on HDAC4 role in the membrane repair pathway. First, we monitored the protein expression levels of two molecular markers in mdx;HDAC4mKO and mdx mice at 6 weeks. A significant reduction in dysferlin and Trim72 protein levels was detected and quantified by WB analyses in mdx;HDAC4mKO GA muscles, compared to mdx ones (Figure 18A). Moreover, IF analyses revealed that while both dysferlin and Trim72 proteins mainly localized in the proximity of the muscle

membrane in mdx GA muscles, this pattern appeared compromised in mdx;HDAC4mKO GA muscles (Figure 18B).

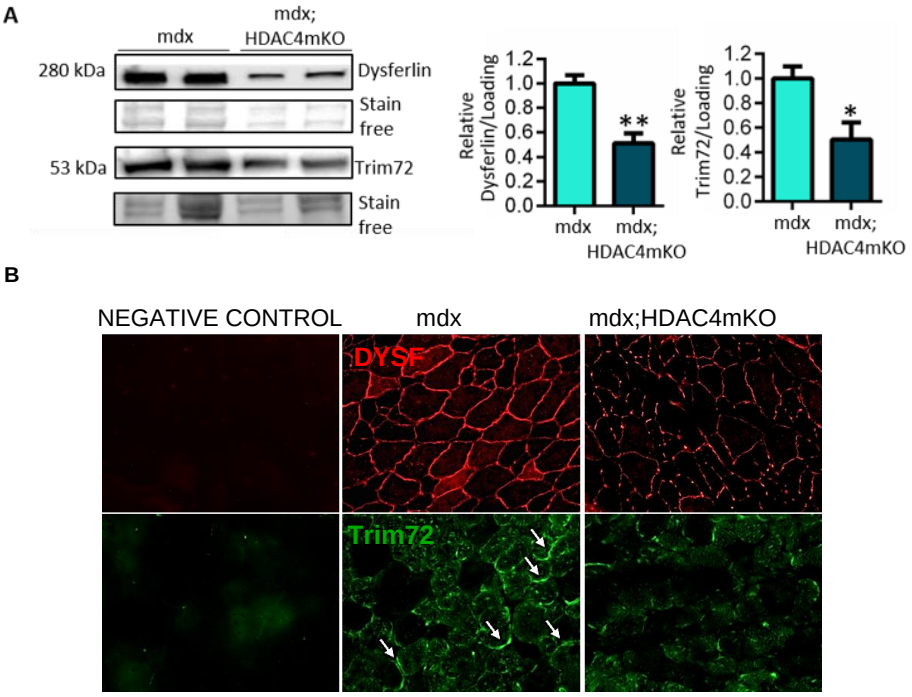
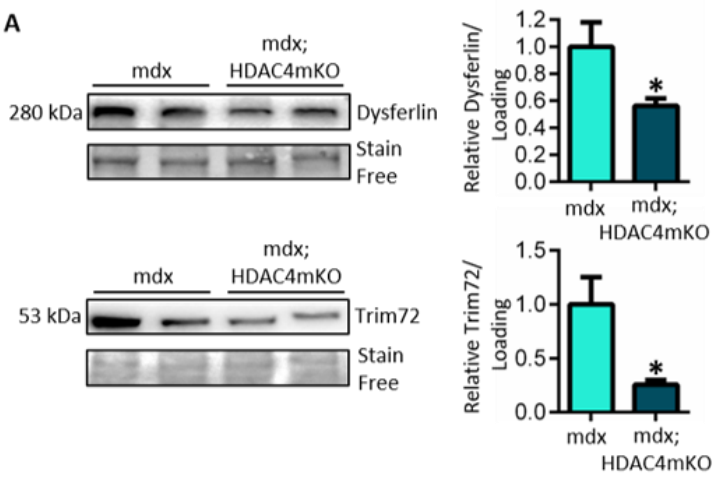



***Figure 18. HDAC4 is necessary for a proper expression and localization of proteins involved in the membrane repair mechanism in mdx muscle***
**A)** Representative western blot and densitometric analyses of dysferlin and Trim72 protein levels in mdx and mdx;HDAC4mKO GA muscles, at 6 weeks of age. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over mdx mice. n=6 mice for each genotype. **p<0.005 by Student's t test. **B)** Representative images of mdx and mdx;HDAC4mKO GA muscle at 6 weeks of age, labelled Dysferlin (red) or Trim72 (green). Scale bar: 50 μm.

We then wondered if defects in the membrane repair mechanism were also reproduced in primary cultures in vitro. SCs were isolated from mdx and mdx;HDAC4mKO mice and dysferlin and Trim72 protein levels were quantified by WB analyses in terminally differentiated myotubes. Both the proteins resulted significantly reduced in mdx;HDAC4mKO myotubes compared to mdx ones (Figure 19A). Coherently, IF analyses confirmed that while both dysferlin and Trim72 proteins were highly expressed in mdx myotubes (Figure 19B) or myofibers (Figure 19C), their expression was very low in mdx;HDAC4mKO cells. Moreover, in mdx cells both the proteins localized in definite patches, probably accomplishing their functions in the membrane repair mechanism, while this specific localization was lost in mdx;HDAC4mKO myotubes or myofibers.



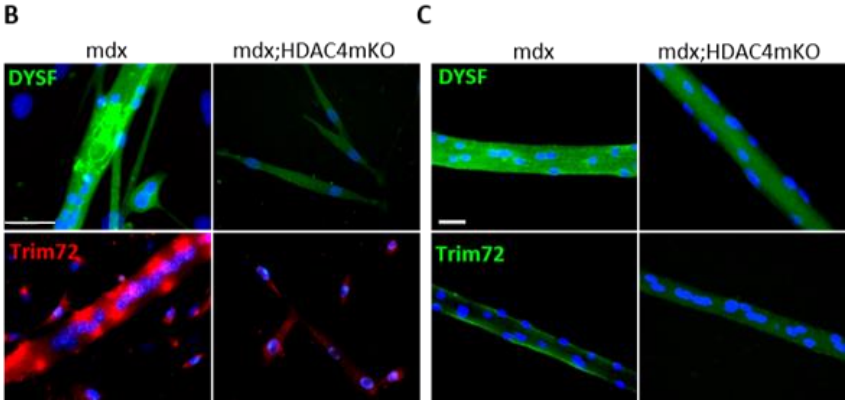



***Figure 19. HDAC4 is necessary for a proper expression and localization of proteins involved in the membrane repair mechanism in mdx myotubes and fibers***

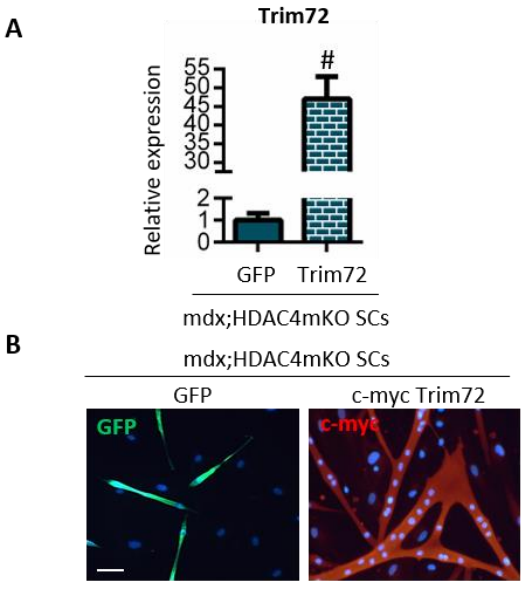
**A)** Representative western blot and densitometric analyses of dysferlin and Trim72 protein levels in mdx and mdx;HDAC4mKO terminally differentiated myotubes. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over mdx mice. n=3 mice for each genotype. *p<0.05 by Student's t test. **B)** Representative images of mdx and mdx;HDAC4mKO terminally differentiated myotubes labelled with Trim72 (red) or dysferlin (green). Scale bar: 50 μm. **C)** Representative images of mdx and mdx;HDAC4mKO myofibers labelled with Trim72 (green) or dysferlin (green). Scale bar: 50 μm.

Overall, these data indicate that HDAC4 is necessary to counteract myofiber death in dystrophic muscles thereby modulating the expression and localization of proteins involved in the membrane repair response.

*4.6 Ectopic expression of Trim72 is sufficient to rescue the mdx;HDAC4mKO phenotype in vitro and in vivo*

We found an aberrant response of the membrane repair mechanism in mdx;HDAC4mKO mice, both *in vivo* and *in vitro*, that probably underpins the higher membrane fragility and the reduced SC

potential. Most of the proteins involved in the membrane repair mechanism are also important for proper myoblast fusion and myogenic potential [223–225]. For instance, Trim72 is an essential component of the cell membrane repair machinery and its knock-down results in compromised myoblast differentiation[113]. Considering that Trim72 protein expression resulted reduced in mdx;HDAC4mKO myotubes, we hypothesized that impaired cell membrane repair leads to decreased differentiation of mdx;HDAC4mKO SCs. To improve membrane repair, we transfected mdx;HDAC4mKO SCs with a c-myc-tagged Trim72-expressing plasmid, or GFP-expressing plasmid as control (Figure 20A) and analyze SC myogenic potential. Ectopic expression of Trim72 significantly improved mdx;HDAC4mKO SC fusion ability and number, compared to GFP-transfected ones (Figure 20B). Increased myogenic ability of Trim72-transfected mdx;HDAC4mKO SCs was corroborated by eMHC and MCK gene expression, quantified by real-time PCR (Figure 20C).



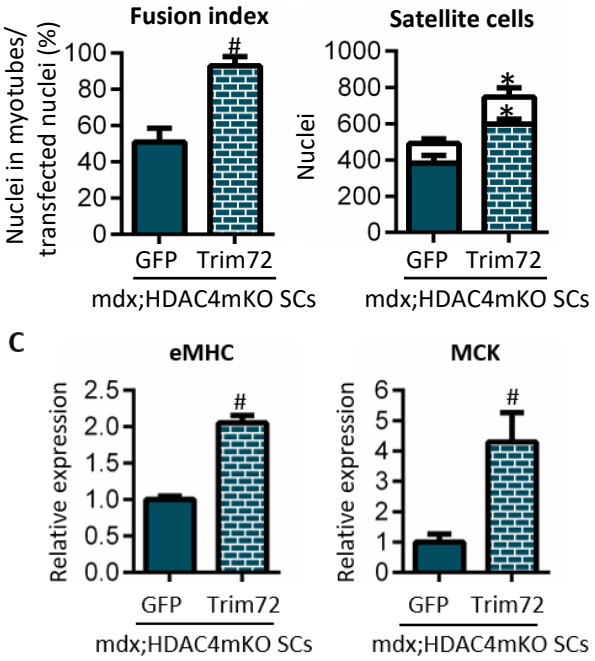



***Figure 20. Ectopic expression of Trim72 is sufficient to rescue mdx;HDAC4mKO satellite cell phenotype***
**A)** Real-time PCR for Trim72 expression in mdx;HDAC4mKO SCs transfected with c-myc-tagged Trim72-expressing plasmid, compared to GFP-transfected ones. Data are presented as mean ± SEM. n=4 mdx;HDAC4mKO mice. #p<0.01 by Student's t-test. **B)** Representative images of IF for GFP and c-myc in transfected and terminally differentiated mdx;HDAC4mKO SCs. Scale bar: 50 μm. Quantification of fusion index and number of satellite cells. Data are presented as mean ± SEM. White bars represent untransfected cells. n=4 mdx;HDAC4mKO mice. *p<0.05; #p<0.01 by Student's t test. **C)** eMHC and MCK expression levels in mdx;HDAC4mKO transfected cells with Trim72, compared to GFP-transfected ones, by real-time PCR. Data are presented as mean ± SEM. n=4 mdx;HDAC4mKO mice. #p<0.01 by Student's t-test.

Importantly, ectopic expression of dysferlin in mdx;HDAC4mKO SCs did not improve their fusion or survival (Figure 21), indicating a specific function of Trim72 in the rescue of mdx;HDAC4mKO phenotype. These results are in line with the previous studies

showing that overexpression of Trim72 facilitates sarcolemma repair in response to multiple insults, including muscle dystrophy [110,226–228], while dysferlin overexpression is detrimental for murine skeletal muscle [229].

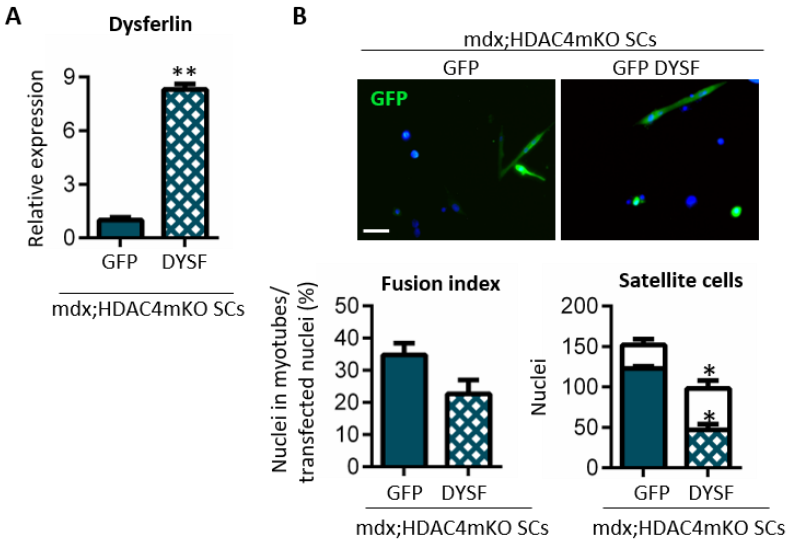



***Figure 21. Ectopic expression of dysferlin does not rescue mdx;HDAC4mKO SCs phenotype***

**A)** Dysferlin expression in mdx;HDAC4mKO SCs transfected with GFP-tagged dysferlin expressing plasmid, compared to GFP-transfected ones, by real-time PCR. Data are presented as mean ± SEM. n=3 mdx;HDAC4mKO mice. **p<0.005 by Student's t-test. **B)** Representative images of IF for GFP in transfected and terminally differentiated mdx;HDAC4mKO SCs. Scale bar: 50 μm. Quantification of fusion index and number of SCs. Data are presented as mean ± SEM. n=4 mdx;HDAC4mKO mice; *p<0.05 by Student's t test.

To evaluate if the higher number of SCs in Trim72-transfected mdx;HDAC4mKO SCs depends on a decrease in cell death, we performed a Tunel assay on mdx;HDAC4mKO transfected SCs. Ectopic expression of Trim72 in mdx;HDAC4mKO SCs leads to a significant decrease in Tunel$^{+}$ nuclei, paralleled to an increase in the

overall number of SCs, compared to GFP-transfected mdx;HDAC4mKO SCs (Figure 22).

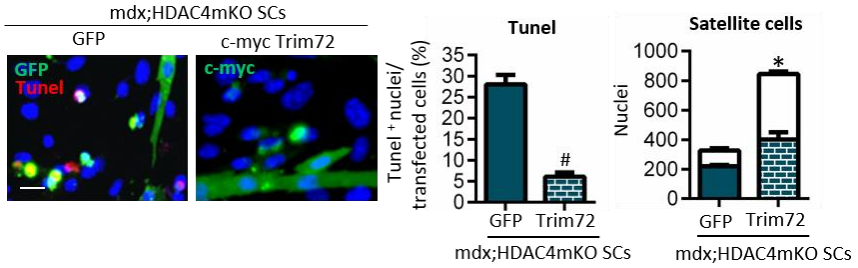



***Figure 22. Ectopic expression of Trim72 improves mdx;HDAC4mKO SCs survival***

Representative images of Tunel assay in differentiating mdx;HDAC4mKO SCs transfected with either GFP or Trim72. Scale bar: 50 μm. Quantification of the Tunel+ cells and number of SCs. Data are presented as mean ± SEM. White bars represent untransfected cells. n=3 mdx;HDAC4mKO mice. *p<0.05; #p<0.01 by Student's t test.

To further prove the involvement of HDAC4 in mediating the membrane repair mechanism response in mdx muscles, we delivered c-myc-tagged Trim72-expressing plasmid, or GFP-expressing plasmid as control, in mdx;HDAC4mKO GA muscles, at 5.5 months of age, by electroporation. First, we confirmed efficiency in gene delivery by IF for GFP and c-myc and by quantifying Trim72 protein levels by WB analyses (Figure 23).

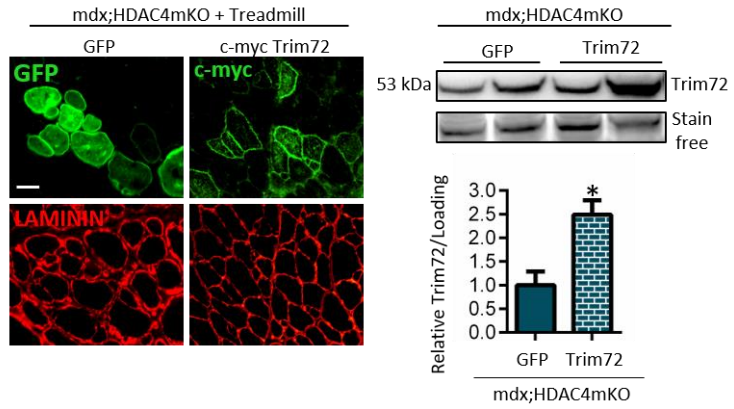
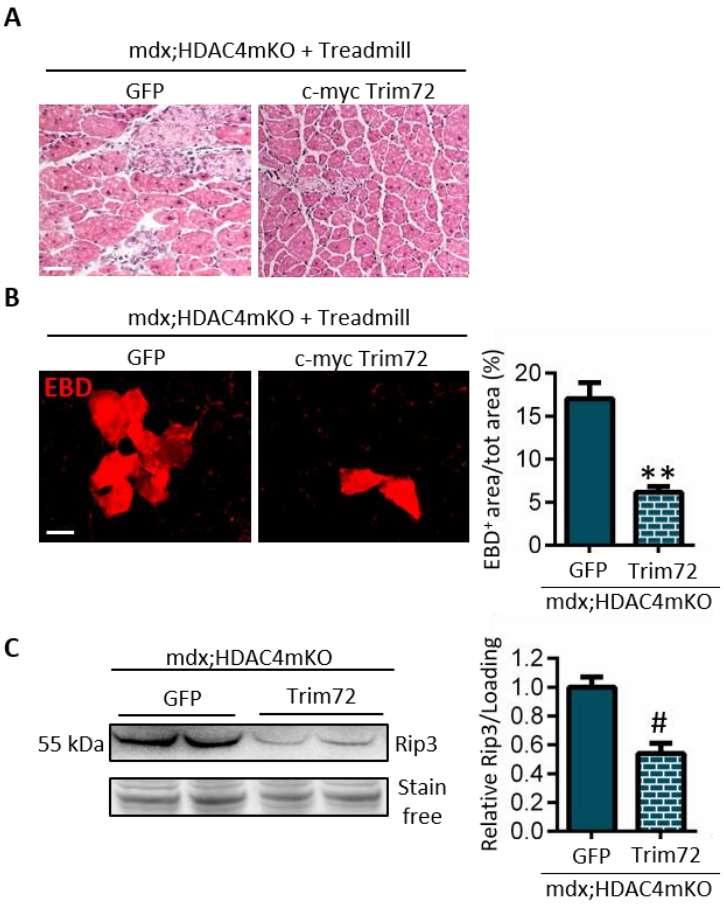



***Figure 23. Efficient gene delivery in mdx;HDAC4mKO GA muscles via electroporation in vivo***
Representative images of IF for GFP and c-myc in mdx;HDAC4mKO GA muscles electroporated with either c-myc-tagged Trim72 or GFP, as control. Scale bar: 50 μm. Trim72 expression in mdx;HDAC4mKO electroporated GA muscles, by western blot analysis. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over GFP-electroporated muscles. n = 4/5 muscles per condition. *p<0.05 by Student's t test.

Two weeks after the electroporation, mice were subjected to the down-hill exercise protocol to exhaustion and muscle phenotype, in terms of muscle morphology, necrosis and functionality was evaluated. Histological analyses of electroporated muscles revealed that ectopic expression of Trim72 apparently decreases muscle necrosis and sustains muscle morphology in mdx;HDAC4mKO muscles (Figure 24A).

Evaluation and quantification of EBD+ fibers proved that delivery of Trim72 significantly reduced the amount of necrotic fibers in mdx;HDAC4mKO muscles, in respect to GFP-electroporated ones (Figure 24B). Decrease in muscle necroptosis was further demonstrated by WB analyses for Rip3 protein levels, which results significantly reduced in the Trim72-electroporated mdx;HDAC4mKO muscles, compared to GFP-electroporated ones

(Figure 24C). Moreover, Trim72 ectopic expression is sufficient to restore dysferlin protein levels and localization in mdx;HDAC4mKO muscles, as proved WB and IF analyses (Figure 24D), in line with a previous study[116].



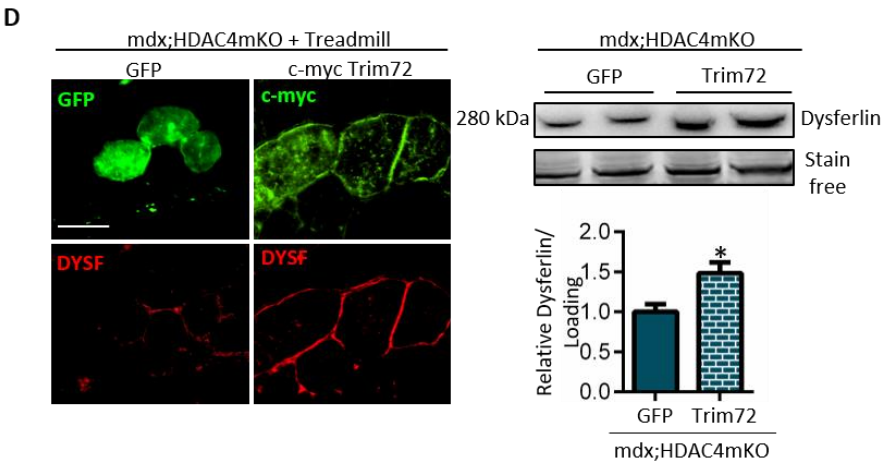



***Figure 24. Ectopic expression of Trim72 preserves muscle homeostasis in mdx;HDAC4mKO mice***

**A)** Representative images of mdx;HDAC4mKO GA muscles electroporated with either c-myc-tagged Trim72 expressing vector or GFP one, as control, stained with hematoxylin and eosin. Scale bar: 50 μm. **B)** Representative pictures and quantification of EBD+ cross sectional area. Scale bar: 50 μm. Data are expressed as mean +/− SEM. n=4 mice per condition. **p<0.005 by Student's t test. **C)** Representative WB and densitometric analyses of of Rip3 in mdx;HDAC4mKO electroporated GA muscles. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over GFP-electr oporated muscles. n = 4 muscles per condition. #p<0.01 by Student's t test. **D)** Representative images of IF for GFP (green), c-myc (green) and dysferlin (DYSF) (red) on mdx;HDAC4mKO electroporated GA muscles. Scale bar:50 μm. Representative WB and densitometric analysis of dysferlin in mdx;HDAC4mKO electroporated GA muscles. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over GFP-electroporated muscles. n = 4 muscles per condition. *p<0.05 by Student's t test.

Importantly, ectopic expression of Trim72 in mdx;HDAC4mKO GA muscles was sufficient to restore the whole muscle functionality, since mdx;HDAC4mKO mice electroporated with Trim72 run longer and further than that one electroporated with GFP (Figure 25).

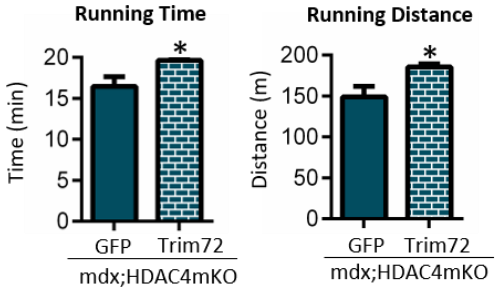



***Figure 25. Ectopic expression of Trim72 restores muscle functionality in mdx;HDAC4mKO mice***
Muscle performance of mdx;HDAC4mKO mice electroporated with either c-myc-tagged Trim72 expressing vector or GFP one, evaluated by treadmill test. Data are presented as mean +/− SEM. n=4 mice per condition. *p<0.05 by Student's t test.

These data suggest that an improvement in the sarcolemma repair mechanism ameliorates mdx phenotype when HDAC4 is deleted in skeletal muscle.

Our data indicate that a defective membrane repair mechanism leads to higher cell death paralleled to compromised SC differentiation, and higher muscle necrosis *in vivo*, since ectopic expression of Trim72 restores mdx;HDAC4mKO SC survival and myogenic potential *in vitro*, and improved muscle homeostasis and function *in vivo*. Trim72 is a cardiac and skeletal muscle-specific TRIM-family protein that identifies and binds phosphatidylserines at the membrane disruption sites and increases membrane resealing of multiple cell types and organs when systemically delivered [230,231]. Also, defects in the membrane repair mechanism associated with mutation in Trim72 cannot be rescued by dysferlin over-expression[232], indicating specific functions for these two proteins. Furthermore, ectopic expression of Trim72 protects skeletal muscle from apoptosis and damage in a genetic muscular dystrophy hamster

model[116], proposing the administration of Trim72 as possible treatment for DMD condition[110].
While overexpression of Trim72 in skeletal muscle induces global insulin resistance and metabolic disorders in mice[233,234], along with cardiomyopathy[235], and controversial role for Trim72 in myogenesis has been reported[223,234], these results were obtained in healthy mice, altering the physiological levels of Trim72 protein.
We believe that the effects of ectopic expression or exogenous delivery of recombinant Trim72 protein may be beneficial in dystrophic conditions, as already demonstrated in mouse and hamster genetic models. Coherently, a recent paper clearly shows the Trim72 protective role in inflammatory myopathies[236]. Patients sera presented elevated levels of Trim72 autoantibodies, able to suppress sarcolemma resealing, contributing to the disease's progression. Indeed, depletion of Trim72 antibodies from serum samples rescued sarcolemma repair capacity.

### *4.7 Ectopic expression of cytoplasmic-restricted HDAC4 ameliorates mdx;HDAC4mKO phenotype in vitro and in vivo*

Being HDAC4 mainly localized in the cytoplasm in adult mdx muscles and considering that activated mdx;HDAC4mKO SCs do express HDAC4 until myogenin expression turns on Cre recombinase, we speculated on a cytoplasmic function of HDAC4 in mdx;HDAC4mKO mice.
To verify this hypothesis, we overexpressed the mutant cytoplasmic form of HDAC4, GFP tagged- HDAC4-L175A[25], wild-type GFP-tagged HDAC4 and the nuclear HDAC4-3SA mutant[25,43], or GFP-expressing plasmid as control, in proliferating mdx;HDAC4mKO SCs and let them differentiate. We confirmed HDAC4 over-expression by real-time PCR (Figure 26A) and analyzed SC fusion and survival. Ectopic expression of cytoplasmic HDAC4 (HDAC4 L/A) greatly increased mdx;HDAC4mKO SC fusion and number (Figure 26B). Of note, neither ectopic expression of wild-type HDAC4, nor the expression of nuclear HDAC4-3SA increased

mdx;HDAC4mKO SC fusion or survival (Figure 26B).

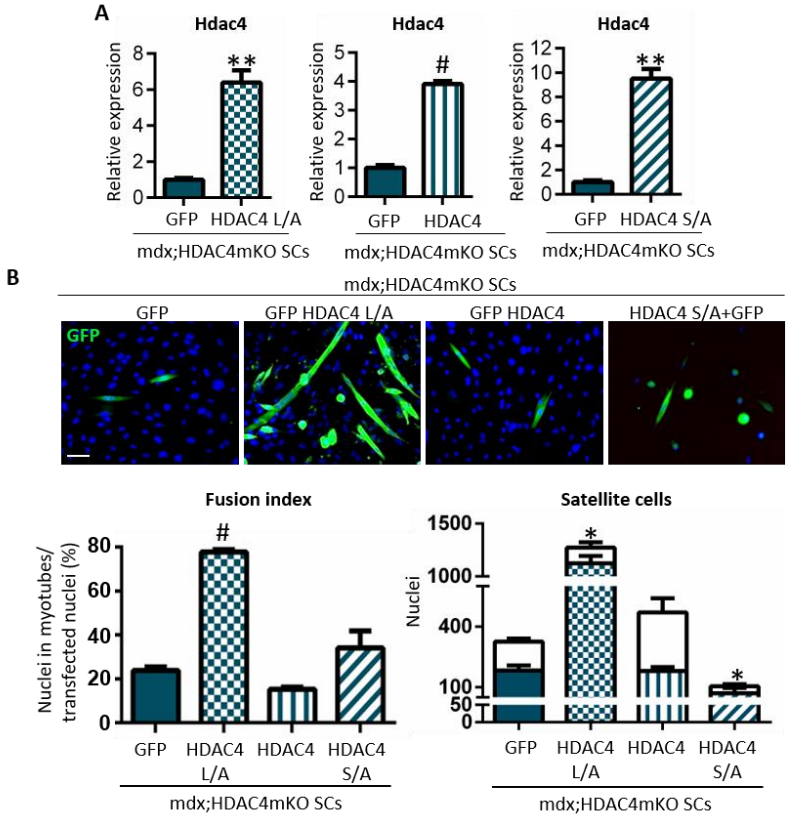



***Figure 26. Ectopic expression of cytoplasmic-restricted HDAC4 ameliorates mdx;HDAC4mKO phenotype in vitro***

**A)** Hdac4 expression in mdx;HDAC4mKO SCs transfected with HDAC4 L/A-, HDAC4-, or HDAC4 S/A-expressing plasmids over GFP-transfected ones, by real-time PCR. Data are presented as mean ± SEM. n=3 mdx;HDAC4mKO mice; #p<0.01; **p<0.005; by Student's t-test. **B)** Representative images of IF for GFP in mdx;HDAC4mKO transfected SCs, after three days of differentiation. Scale bar: 50 μm. Quantification of fusion index and number of satellite cells. n=3 mdx;HDAC4mKO mice. Data are presented as mean ± SEM. White bars represent untransfected cells. One-way ANOVA reveals a significant effect and interaction: *p<0.05; #p<0.01 versus GFP by Tukey's HSD test.

Our results are in line with previous data shown in literature. Indeed, wt HDAC4, as well as the mutant nuclear HDAC4 overexpression, inhibits myoblast differentiation in vitro and exert deleterious effects on skeletal muscle in vivo [237,238]. Indeed, HDAC4 localizes within the nucleus in proliferating myoblasts, where it associates with and inhibits MEF2-dependent transcription, thereby inhibiting myogenesis. Upon differentiation, class II HDACs translocate into the cytoplasm, thereby releasing the inhibition on MEF2, which promotes myoblast differentiation.
Overall, the transfection experiments clearly indicate that HDAC4 exerts its protective functions in the cytoplasm of dystrophic SCs. Shedding light on HDAC4 cytoplasmic functions will provide a strategy to preserve its protective function without affecting HDAC4 nuclear levels, important for proper myogenesis.

Considering the encouraging data obtained in vitro, we delivered the cytoplasmic c-myc-tagged HDAC4 L/A-expressing plasmid, or GFP-expressing one, in mdx;HDAC4mKO GA muscles, at 5.5 months of age, via electroporation. After two weeks, we subjected mice to the down-hill exercise protocol to exhaustion and evaluated muscle phenotype, in terms of muscle morphology, necrosis and regeneration. Efficiency in gene delivery was determined by IF for GFP and c-myc and quantified by WB analyses for HDAC4 in electroporated muscles (Figure 27).

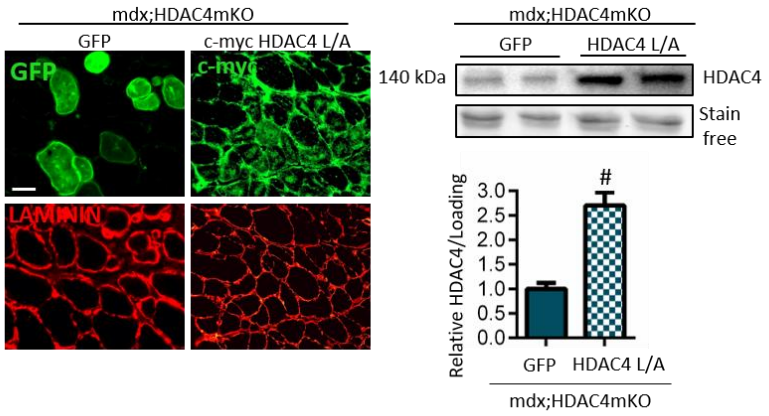



***Figure 27. Efficient ectopic expression of HDAC4 L/A in mdx;HDAC4mKO GA muscles via electroporation***
**A)** Representative images of IF for GFP and c-myc in mdx;HDAC4mKO GA muscles electroporated with either c-myc-tagged HDAC4 L/A or GFP, as control. Scale bar: 50 um. **B)** HDAC4 expression in mdx;HDAC4mKO electroporated GA muscles by WB analysis. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over GFP-electroporated muscles. n=4/5 muscles per condition. #$p<0.01$ by Student's t test.

Histological analyses revealed that ectopic expression of cytoplasmic HDAC4 apparently decrease muscle necrosis and improved regeneration in mdx;HDAC4mKO muscles (Figure 28A).

Evaluation and quantification of EBD+ fibers proved that delivery of cytoplasmic HDAC4 significantly reduced the number of necrotic fibers in mdx;HDAC4mKO muscles, with respect to GFP-electroporated ones (Figure 28B). Decrease in muscle necroptosis was further demonstrated by WB analyses for Rip3 protein levels, which results significantly reduced in the HDAC4 L/A-electroporated mdx;HDAC4mKO muscles, compared to GFP-electroporated ones (Figure 28C). Increased muscle regeneration was confirmed by higher myogenin and eMHC protein levels in HDAC4 L/A-electroporated muscles, in respect of GFP-electroporated ones (Figure 28D)

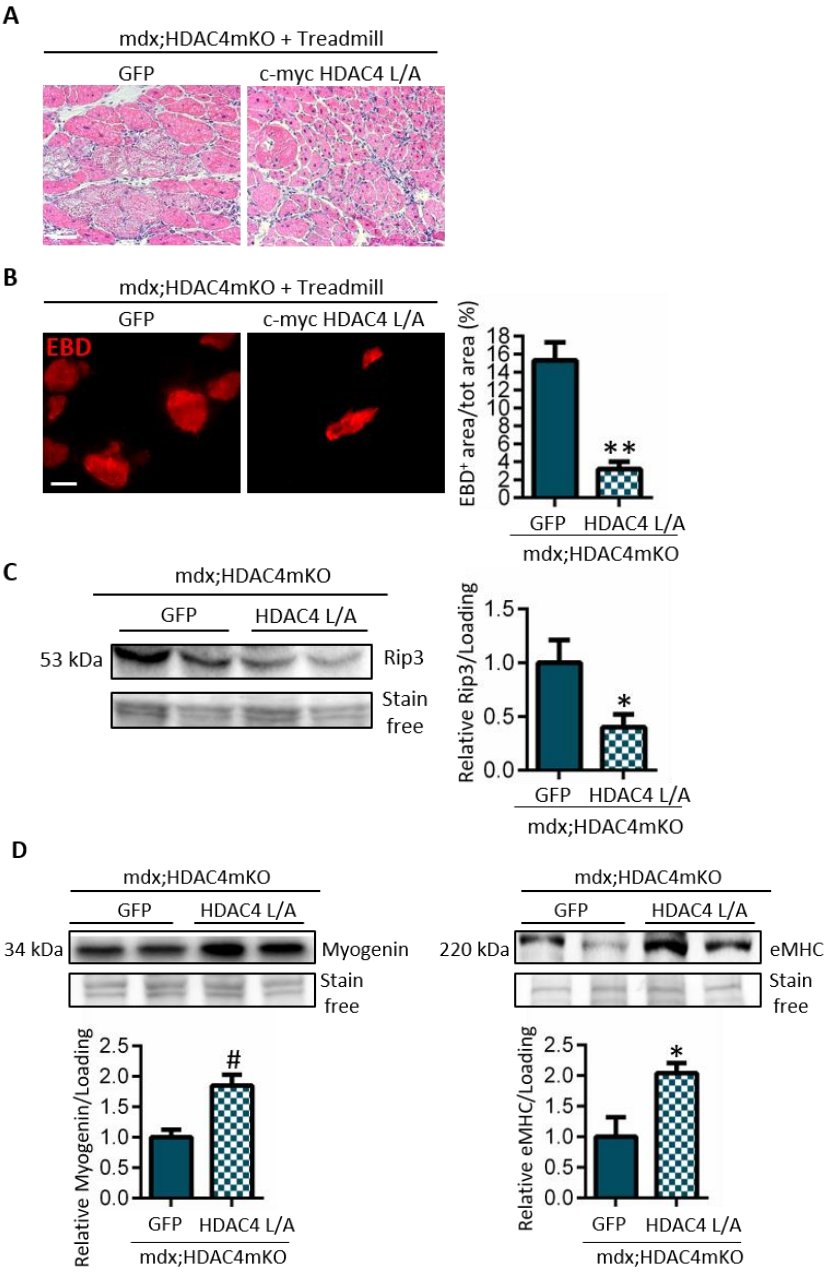

A
mdx;HDAC4mKO + Treadmill
GFP
c-myc HDAC4 L/A
B
mdx;HDAC4mKO + Treadmill
GFP
c-myc HDAC4 L/A
EBD
EBD+ area/tot area (%)
18
16
14
12
10
8
6
4
2
0
**
GFP HDAC4 L/A
mdx;HDAC4mKO
C
mdx;HDAC4mKO
GFP
HDAC4 L/A
53 kDa
Rip3
Stain free
Relative Rip3/Loading
1.5
1.0
0.5
0.0
*
GFP HDAC4 L/A
mdx;HDAC4mKO
D
mdx;HDAC4mKO
GFP
HDAC4 L/A
34 kDa
Myogenin
Stain free
Relative Myogenin/Loading
2.5
2.0
1.5
1.0
0.5
0.0
#
GFP HDAC4 L/A
mdx;HDAC4mKO
mdx;HDAC4mKO
GFP
HDAC4 L/A
220 kDa
eMHC
Stain free
Relative eMHC/Loading
2.5
2.0
1.5
1.0
0.5
0.0
*
GFP HDAC4 L/A
mdx;HDAC4mKO

***Figure 28. Ectopic expression of cytoplasmic-restricted HDAC4 ameliorates mdx;HDAC4mKO phenotype in vivo***
**A)** Representative images of mdx;HDAC4mKO GA muscles electroporated with either HDAC4 L/A expressing vector or GFP, as control, stained with hematoxylin and eosin. Scale bar: 50 μm. **B)** Representative pictures and quantification of EBD+ cross sectional area. Scale bar: 50 μm. Data are expressed as mean +/− SEM. n=4 mice per condition. **p<0.005 by Student's t test. **C)** Representative WB and densitometric analyses of Rip3 protein levels in mdx;HDAC4mKO electroporated GA muscles. Stain-free protein bands were used as a loading control. Data are expressed as mean +/− SEM over GFP-electroporated muscles. n=4 mice per condition. *p<0.05 by Student's t test. **D)** Representative WB for myogenin and eMHC in mdx;HDAC4mKO electroporated GA muscles with densitometric analyses. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM over GFP-electroporated muscles. n=4/5 muscles per condition. *p<0.05; #p<0.01 by Student's t test.

Importantly, ectopic expression of cytoplasmic HDAC4 in GA muscles was sufficient to restore whole muscle functionality, since mdx;HDAC4mKO mice electroporated with HDAC4 L/A expressing plasmid run longer and further than that one electroporated with GFP-expressing one (Figure 29).

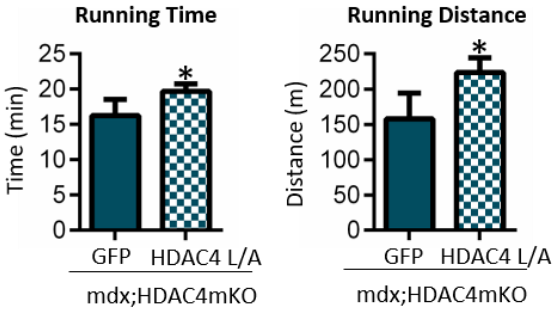



***Figure 29. Ectopic expression of cytoplasmic-restricted HDAC4 improves muscle performance in mdx;HDAC4mKO mice***
Muscle performance of mdx;HDAC4mKO mice electroporated with either HDAC4 L/A-expressing vector or GFP, as control, by treadmill test. Data are

presented as mean +/− SEM. n=4/5 mice per condition. *p<0.05 by Student's t test.

Eventually, ectopic expression of cytoplasmic HDAC4 induces higher expression of dysferlin and Trim72 proteins as proved by WB analysis (Figure 30), important for restoring proper membrane repair mechanism.

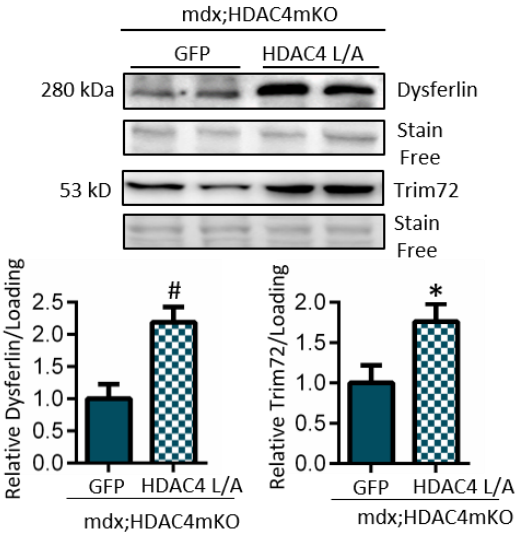



***Figure 30. Ectopic expression of cytoplasmic-restricted HDAC4 restores dysferlin and Trim72 protein expression in mdx;HDAC4mKO mice***
Representative WB and densitometric analysis of dysferlin and Trim72 in mdx;HDAC4mKO electroporated GA muscles. Stain-free protein bands were used as a loading control. Data are shown as mean ± SEM, over GFP-electroporated muscles. n=4 muscles per condition. #p<0.01; *p<0.05 by Student's t test.

### *4.8. Cytoplasmic HDAC4 colocalizes with Trim72 in mdx myotubes*

Based on the result that HDAC4 L/A ectopic expression restores Trim72 protein levels in mdx;HDAC4mKO mice, we speculated a possible HDAC4-Trim72 protein interaction in the cytoplasm

responsible for stabilizing Trim72 protein. To assess this hypothesis, we performed a double-immunolabeling with HDAC4 and Trim72 primary antibodies of terminal differentiated mdx myotubes and immunofluorescences were detected with a confocal laser scanning fluorescence microscope. HDAC4-Trim72 protein colocalization was observed (Figure 31), thus suggesting a possible protein-protein interaction.

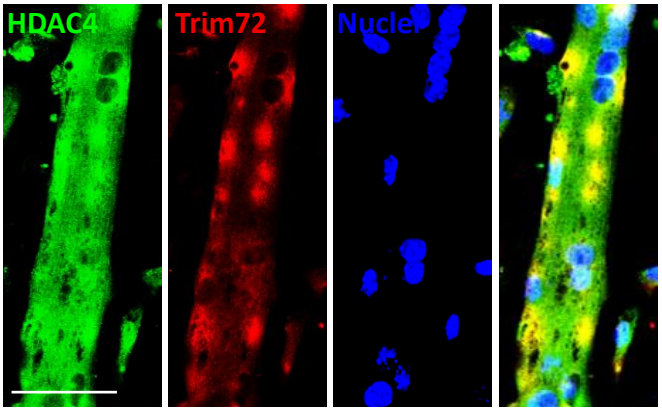



***Figure 31. HDAC4 and Trim72 protein colocalization in mdx myotubes***
Representative images of mdx terminally differentiated myotubes double labelled with HDAC4 (green) and Trim72 (red) by confocal microscope. Nuclei were stained in blue. Scale bar: 50 μm.

### *4.9 The protective role of cytoplasmic HDAC4 is independent of its deacetylase activity in dystrophic muscles*

To further investigate the molecular mechanisms, we wondered whether HDAC4 protective cytoplasmic functions in dystrophic muscle were mediated by its deacetylase activity. In collaboration with Prof. Claudio Brancolini's group, a mutant cytoplasmic form of HDAC4 with an asparagine (N) for aspartic acid (D) substitution in the active site (D840N), which renders HDAC4 catalytically dead[39,239], was generated. We overexpressed the mutant GFP-tagged HDAC4 L/A D840N, in proliferating mdx;HDAC4mKO SCs, or

GFP-expressing plasmid as control, and analyze SC fusion and number. Ectopic expression of the mutant HDAC4 L/A D840N greatly increased mdx;HDAC4mKO SC fusion and number (Figure 32), similarly to HDAC4 L/A overexpression, indicating that HDAC4 protective functions in mdx SCs are independent of its deacetylase activity.

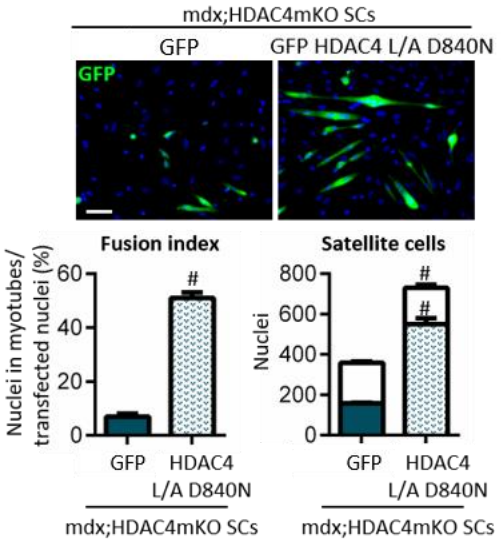



***Figure 32. Ectopic expression of a catalytically dead cytoplasmic-restricted HDAC4 mutant retains the HDAC4 protective functions in mdx;HDAC4mKO SC***

Representative images of IF for GFP in mdx;HDAC4mKO SCs transfected with either GFP-tagged HDAC4 L/A D840N or GFP, as control, after three days of differentiation. Scale bar: 50 µm. Quantification of fusion index and number of satellite cells. n=3 mdx;HDAC4mKO mice. Data are presented as mean ± SEM. White bars represent untransfected cells. *p<0.05; #p<0.01 by Student's t test.

Recently, a connection between HDACs and muscle fragility in DMD has been published[240]. However, in this study pan-HDACi have been used as epigenetic regulators while cytoplasmic/non-deacetylase functions of class II HDACs were not investigated. Non-catalytic roles of HDAC4 have been proposed in the literature,

including acting as acetyl-lysine reader to support HDAC3 deacetylase activity or simply as a scaffolding protein in the nucleus[239]. Recently, cytoplasmic deacetylase activity of HDAC4 in skeletal muscle has been reported upon denervation[57]. However, the possibility that HDAC4 modulates cytoplasmic protein activities through "unconventional functions" cannot be excluded.
Our results indicate a possible HDAC4-Trim72 protein interaction which may enhance Trim72 protein stability through a deacetylase-independent mechanism. To date, only one Trim72 post-translational modification is reported to influence protein stability, i.e. S-nitrosylation at Cys-144, which protects Trim72 against oxidation-induced protein degradation and cell death[241]. Moreover, acetylation sites were not reported in Trim72 protein. Further studies are currently on going to prove a direct physical interaction between HDAC4 and Trim72 protein in mdx muscles.

Taking into account the protective role of HDAC4 in the cytoplasm, in addition to confirm HDAC4 activities other than deacetylation, future investigations will be focused on possible pharmacological approaches to promote HDAC4 cytoplasmic retention. For instance, interfering with the nuclear-cytoplasmic shuttling by inhibiting HDAC4 dephosphorylation may be considered as a therapeutic approach for preclinical studies. Otherwise, administration of HDAC4-cytoplasmic restricted form by viral infection or recombinant protein injection could be considered.

To conclude, we proved that HDAC4 is important for maintaining skeletal muscle integrity in mdx mice. HDAC4 deletion in mdx skeletal muscles exacerbates muscle degeneration, increases circulating creatine kinase levels, decreases muscle regeneration and functionality over time. Moreover, SC biology is affected in mdx;HDAC4mKO mice in terms of impaired myogenic potential and survival. In addition to directly regulate SC by cell-autonomous mechanisms, HDAC4 modulates SC niche remodeling by releasing soluble factors and affecting FAP behavior.

In addition, we reported worsened membrane fragility in mdx;HDAC4mKO mice and an impaired membrane repair response *in vitro* and *in vivo* that in part underpin the more pronounced progression of the pathology.
Coherently, the ectopic expression of Trim72, a main player in the membrane repair response, is sufficient to improve mdx;HDAC4mKO muscle homeostasis, thus suggesting HDAC4 involvement to maintain membrane stability.
Further investigations revealed that cytosolic HDAC4 mediates membrane repair mechanism response *in vitro*, allowing SC survival and differentiation, and *in vivo*, ameliorating membrane fragility, promoting *de novo* myogenesis and improving overall muscle function in mdx;HDAC4mKO mice.
Eventually, HDAC4 deacetylase activity seems to be not responsible for HDAC4 protective function in mdx SCs, suggesting alternative cytosolic functions of HDAC4.
These findings shed new light on the cytoplasmic role of HDAC4 in skeletal muscle, not completely yet characterized.

## 5. Materials and Methods

*5.1 Plasmids*

The following plasmids were used: Snap-GFP (generously provided by Tullio Pozzan); pcDNA3.1 myc/His (Thermofisher); Flag-HDAC4 (Addgene, #13821); pcDNA-hHDAC4 [NM_006037.3]*L175A/Myc (named HDAC4 L/A) (CliniSciences); GFP-hHDAC4 [NM_006037.3]*L175A D840N (named GFP HDAC4 L/A D840N) (generated by Claudio Brancolini's laboratory); pcDNA-HDAC4.3SA-FLAG (Addgene, #30486) (named HDAC4 S/A); EGFP-Dysferlin (kindly provided by Simone Spuler); Trim72 (Myc-DDK-tagged) (OriGene).

*5.2 Mice*

HDAC4 conditional mutant mice were generously provided by Prof. Eric N Olson and Rhonda Bassel-Duby[55]. To study the role of HDAC4 in DMD, Hdac4$^{fl/fl}$ myogenin;Cre mice were crossed with the mdx transgenic mice (Charles River Laboratories), obtaining mdx;Hdac4$^{fl/fl}$ myogenin;Cre mice (named mdx;HDAC4mKO mice) and mdx;Hdac4$^{fl/fl}$ mice (referred to as mdx mice). In all the experiments, female mdx;HDAC4mKO and mdx littermates were compared. In addition, aged-matched, female Hdac4$^{fl/fl}$ mice (referred to as CTR mice) were used as control healthy mice.

*5.3 Ethics statement*

Mice were treated in strict accordance with the guidelines of the Institutional Animal Care and Use Committee, as well as national and European legislation, throughout the experiments. Animal protocols were approved by the Italian Ministry of Health (authorization # 853/2016-PR).

*5.4 Functional analyses*

The treadmill test was performed by using the Exer-6M (Columbus Instruments) with a down-hill exercise protocol to exhaustion, as previously described in[242]. For 16 month-old mice, the protocol was adapted as previously reported in[214].

*5.5 DNA delivery by electroporation*
In anesthetized mice, GA muscles were exposed, injected with 12 μg of DNA in 5% mannitol solution directly in two of the three muscle lobes and immediately subjected to electric stimulation using a pulse generator (ECM 830, BTX), equipped with 3 X 5-mm genepaddle electrodes, placed at opposite sides of the muscle. Electroporation was performed by delivering six electric pulses of 10V each, with a fixed duration of 20 ms and an interval of 200 ms between the pulses, as in[243]. In preliminary experiments, GFP-expressing vector was co-electroporated with myc-tagged expressing vectors in 1:3 ratio, resulting in colocalization of GFP and c-myc in most of the electroporated fibers. We therefore pursued the analyses by delivering c-myc tagged expressing vectors for the protein of interest and GFP-expressing plasmid as control.

*5.6 Serum withdrawal and CK assay*
Serum samples were collected from blood drawn via cardiac puncture from 6 week-old mice and stored at –80°C until used. Serum was assayed using a colorimetric Creatine Kinase Activity Assay Kit, according to the manufacturer's protocol (RayBiotech).

*5.7 Sarcolemma Membrane Integrity by Evans Blue Dye Uptake*
Mice were intraperitoneally injected with 10 μl/g body weight of a 1% Evan's Blue Dye (EBD) solution in phosphate-buffered saline (Sigma-Aldrich) 8 hours before the sacrifice. Animals were sacrificed and muscles were frozen in liquid nitrogen-cooled isopentane. EBD signal was recorded on formaline-fixed sections by using a 516/560 excitation/emission pair.

*5.8 Histological analyses*
GA muscles were dissected, embedded in tissue freezing medium (Leica), and frozen in isopentane pre-cooled with liquid nitrogen. Serial sections (9 μm) of the mid-belly of GA muscles were obtained by using a Leica cryostat. Hematoxylin and eosin (H&E) (Sigma-

Aldrich) was performed according to the manufacturer's instructions.

*5.9 Immunofluorescences*

Cryosections or cell plates were fixed in formalin buffered solution 10% (Sigma-Aldrich) for 10 minutes at room temperature, then washed and blocked with 1% BSA (Sigma-Aldrich) for 30 min or with 10% goat serum for 30 min. Samples were then incubated with one of the following antibodies, diluted in 1% BSA: 1:50 rabbit polyclonal anti-laminin antibody (Sigma-Aldrich), 1:400 mouse/rabbit mono o poly anti-GFP (marca), 1:50 c-Myc antibody idem complete, 1:40 anti-eMHC antibody (Hybridoma Bank), 1:100 anti-Dysferlin antibody (Abcam), 1:80 anti-Trim72 antibody (Abcam), 1:10 sarcomeric MHC antibody (clone MF 20, Developmental Studies Hybridoma Bank) overnight at 4 °C. To detect the primary antibodies, incubation with a 1:500 dilution of anti-rabbit-Alexa 488 or Alexa555 (Thermo Fisher Scientific) secondary antibodies in 1% BSA for 1 hour at room temperature or with 1:1000 biotin-conjugated secondary antibody (Jackson ImmunoResearch) for 45 minutes at room temperature, followed by 1:2500 streptavidin antibody (Jackson ImmunoResearch) for 30 minutes at room temperature was performed. For IgG staining, after blocking, muscles were incubated with a 1:500 dilution in 1% BSA of anti-mouse-Alexa 555 (Thermo Fisher Scientific) secondary antibody, for 1 h at room temperature. Additional step of permeabilization was used for c-myc (1% BSA/0.2% Triton/PBS for 30 min), and alternative fixation with cold acetone for 5' at -20°C for eMHC, or cold methanol for 5' at -20°C for dysferlin and trim72 were used. 0.5 µg/ml Hoechst 33,342 (Sigma-Aldrich) was used to stain nuclei. Samples were mounted with 60% glycerol in Tris HCl 0.2 M pH 9.3.

For confocal analysis, the following secondary antibodies were used: Cy5 AP donkey anti–rabbit IgG 1:200 (Cy5_emission maximum 669 nm) Jackson ImmunoResearch Laboratories, and goat anti-mouse-Alexa 488.

Photographs were acquired using an Axio imager A2 system equipped with an Axiocam HRc, with Axiovision Release 4.8.2 software (Zeiss) or by using Leica Confocal Microscope for double-immunolabeling, Z-stack images were obtained at sequential focal planes 0.5 μm apart by confocal microscope (Laser Scanning TCS SP2; Leica). using a HCX PL APO CS 40.0x1.25 OIL objective, with a 2x digital zoom.

*5.10 Morphometric analyses*

Photographs were acquired using an Axio imager A2 system equipped with an Axiocam HRc, with Axiovision Release 4.8.2 software (Zeiss), at standard 1300 × 1030 pixel resolution. Myofiber cross-sectional area was quantified on H&E or immunostained sections from the mid-belly of GA and diaphragm muscles by using Image J software. The entire muscle section of the muscle was quantified for each replicate.

*5.11 Myofiber isolation*

Single myofibers were isolated from EDL muscles of 6-week-old mice as previously described in [216,217].

*5.12 Satellite cell and Fibro Adipogenic Progenitor isolation by GentleMACS and culture conditions*

Satellite cells were isolated from 3-week-old mice also by using MACS microbeads technology (GentleMACS, Miltenyi Biotec). GentleMACS dissociation was performed according to the manufacturer's protocol. Briefly, skeletal muscle was cut into small fragments and put in a C-tube with the enzyme cocktail of the dissociation Kit, according to the manufacturer's recommendation. Muscles were then subjected to a mechanical disaggregation step in the GentleMACS dissociator, with an incubation at 37 °C. After dissociation, samples were filtered to remove any remaining larger particles from the single-cell suspension. Satellite cells were then isolated using the Satellite Cell Isolation Kit, by depletion of non-

target cells, which are magnetically labeled with a cocktail of monoclonal antibodies conjugated with MACS MicroBeads. Satellite cells were plated on 0.01% collagen (Sigma-Aldrich)-coated dishes with Dulbecco's modified Eagle medium supplemented with 20% horse serum (Sigma-Aldrich),100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 3% of chicken embryo extract as growing medium (GM). After 48 hours, the medium was replaced with differentiation medium (DM) (GM diluted 1:10). FAPs cells were isolated from cell suspension depleted of SCs obtained by Satellite Cell Isolation Kit and by using Anti-Sca-1 magnetic MicroBeads (Miltenyi Biotec). Cells were plated on 0.01% collagen (Sigma-Aldrich)-coated dishes with Dulbecco's modified Eagle medium supplemented with 20% horse serum (Sigma-Aldrich),100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 3% of chicken embryo extract as growing medium (GM). For adipogenic differentiation, after 7 days in GM, FAPs were exposed for 3 days to adipogenic induction medium consisting of DMEM with 10% FBS (Gibco), 0.5 mM IBMX (Sigma), 0.25 µM dexamethasone (Sigma) and 10 µg/ml insulin (Sigma), followed by further 3 days in adipogenic maintenance medium, consisting of DMEM with 10% FBS and 10 µg/ml insulin. Cells were stained with Oil Red O (Sigma-Aldrich) following manufacturer's protocol. Cell culture inserts with 1.0-µm pore and 24-well culture plates (Falcon) were used for transwell co-culture. Inserts and plates were coated with 0,1% gelatin. 1 x $10^4$ freshly sorted MuSCs were plated in the bottom of the plate, while 1 x $10^4$ FAPs cells were plated on the upper insert.

*5.13 Cytofluorimetric Analysis*

To prepare single cell suspension, muscles were dissociated by using GentleMACS kit as described above. Then, digested muscles were passed through a 70 µm cell strainer first and then through a 40 µm cell strainer, to exclude cell debris. Muscle single cell suspension was then resuspended in FACS buffer (PBS 1% FBS)

and incubated 30 min on ice with the following antibodies: anti-CD45, anti-Ly6G, anti-Ly6c, anti-F4/80, anti-CD11b and anti-CD3. Cell viability was assessed with 40 ,6-diamidino-2-phenylindole dilactate (DAPI) (BioLegend). Samples were processed using a Dako CyAn ADP flow cytometer and acquired data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA).

*5.14 DNA delivery by transfection*

For transient transfection, $3 \times 10^5$ mdx;HDAC4mKO satellite cells were seeded in 35-mm diameter plates. After 1 hour, cells were transfected with 4 μg of total DNA, using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. In preliminary experiments, GFP-expressing vector was co-transfected with myc-tagged expressing vectors in 1:3 ratio, resulting in colocalization of GFP and c-myc in most of the transfected myotubes. We therefore pursued the analyses by delivering c-myc tagged expressing vectors for the protein of interest and GFP-expressing plasmid as control. When GFP-expressing vector was co-transfected with the plasmid of interest (HDAC4 S/A) in a 1:3 ratio, also FLAG-HDAC4 and GFP-HDAC4 L/A were co-transfected with pcDNA in 3:1 ratio, in order to compare samples with equal amount of plasmid of interest. Transfected cells were cultured in GM for 48 hours and then shifted to DM.

*5.15 Tunel Assay*

Tunel assay was performed using the ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore) following the manufacturer's instructions.

*5.16 RNA extraction and real-time PCR*

Total RNA was isolated and purified from 30 to 50 mg of GA muscles or from cell plates by using Trizol (Invitrogen), following the manufacturer's protocol. One microgram of total RNA was converted to cDNA by using the PrimeScript™ RT reagent Kit

(Takara). Real-time PCR was performed with the SDS-ABI Prism 7500 (Applied Biosystem) by using the TB GreenTM Premix Ex TaqTM mastermix (Takara) and primers listed in Table 1. Gapdh was used as loading control.

Table 1. Primer sequences used in the study.

| Gene | Forward | Reverse |
|---|---|---|
| Hdac4 | GTCTTGGGAATGTACGACGC | GTTGCCAGAGCTGCTATTTG |
| Dysferlin | GAATCCCCTGTTCTCCTCGC | CAAAGCCCTCATTGGACACG |
| Trim72 | GCCTCAAGACACAGCTTCCA | TGCTTCACGGTCCAGAGAAC |
| eMHC | TCGTCTCGCTTTGGCAA | TGGTCGTAATCAGCAGCA |
| MCK | CACCATGCCGTTCGGCAA CA | GGTTGTCCACCCCAGTCT |
| Gapdh | ACCCAGAAGACTGTGGATGG | CACATTGGGGGTAGGAACA |

*5.17 Protein extraction and western blot analyses*

GA muscles were dissected, minced, and homogenized in lysis buffer (50 Mm Tris HCl pH 7.4, 1 mM EDTA, 150 Mm NaCl, 1% Triton) supplemented with protease and phosphatase inhibitors. Alternatively, cells washed and collected in 100 µl of lysis buffer and left on ice for 30 minutes. For protein subcellular fractionation, the protocol described in[244] was used. Protein concentration was quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific). For each sample, 40–70 µg of proteins were run on the nUView Tris-Glycine gels. Gels were transferred to nitrocellulose membranes, and protein bands were visualized under UV light, without staining. The whole signal of the entire lane has been used for loading and transfer control. Membranes were blocked with 5% milk or 5% BSA in TBST and blotted with different primary antibodies. After washing in TBST, membranes were incubated with HRP-conjugated secondary antibodies (BIO-RAD), and signals were detected by using ECL chemistry (Cyanagen).

Images were acquired on a ChemiDoc MP imaging system (BIO-RAD) with Image Lab 5.2.1 software. The following primary antibodies were used: HDAC4 (Santa Cruz), Gapdh (Santa Cruz), Dysferlin (Abcam), Trim72 (Abcam), Rip3 (Abcam), eMHC (Hybridoma bank), Myogenin (Hybridoma bank), H3 (Cell-signaling).

*5.18 HDAC activity assay*

HDAC activity was evaluated by using different fluorogenic substrates specific for class I, class IIa or Class I/IIb HDACs. The assay was performed as previously described in[212]. Briefly, tissue extracts were homogenized in PBS (pH 7.4) containing 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific) using a pellet pestle motor (KONTES) and sonicated prior to clarification by centrifugation. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Tissue extracts were diluted into PBS buffer in 100 µl total volumes in 96-well plate (30 µg GA protein/well). Where indicated, TSA (1 µl of 100× DMSO stock solution) or corresponding volumes of DMSO were added, followed by 45' incubation at 37° C. Substrates were added (5 µl of 1 mM DMSO stock solution), and the plates were returned to the 37°C incubator for 3 hours. Then, developer/stop solution was added (50 µl per well of PBS with 1.5% Triton X-100, 3 µM TSA, and 0.75 mg/ml trypsin), with additional 20' incubation at 37°C. To detect fluorescent signal Glowmax (Promega) instrument with excitation and emission filters of 360 nm and 460 nm, respectively was used. Background signals from buffer blanks were subtracted, and data were normalized as needed using appropriate controls.

*5.19 Statistics and program*

Statistical significance was determined by using two-tailed Student's *t*-test when two conditions were compared, or with one-way analysis of variance (ANOVA), followed by Tukey's HSD test as a post-hoc test, when more than two conditions needed to be

compared. All values were expressed as mean ± standard error of the mean (SEM).

VassarStats, a statistical computation website (http://vassarstats.net/), was used for the statistical analyses. All the artwork was created by using GraphPad Prism 6 program. Differences between mdx and mdx;HDAC4mKO mice were confirmed in independent experiments, i.e., independent litters, by considering as "n" an independent biological sample, not a technical replicate.

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## 7. List of Publications

**1)** Cytoplasmic HDAC4 regulates the membrane repair mechanism affecting muscle homeostasis in Duchenne Muscular Dystrophy.
**Alessandra Renzini**, Nicoletta Marroncelli, Giorgia Cavioli, Silvia Di Francescantonio, Sergio Valente, Antonello Mai, Claudia Giampietri, Sergio Adamo, Dario Coletti, Viviana Moresi. *in preparation.*

**2)** Neurohypophyseal Hormones and Skeletal Muscle: A Tale of Two Faces.
Alexandra Benoni, **Alessandra Renzini**, Giorgia Cavioli, Sergio Adamo. Eur J Transl Myol 2020 Apr 1;30(1):8899. doi: 10.4081/ejtm.2019.8899. eCollection 2020 Apr 7.

**3)** Metabolic control of muscle stem cells.
**Renzini Alessandra**; Adamo, Sergio; Moresi, Viviana. ORGANISMS. - ISSN 2532-5876. - 3:2(2019), pp. 24-35.

**4)** Thyroid Hormone Protects from Fasting-Induced Skeletal Muscle Atrophy by Promoting Metabolic Adaptation.
Ucci Sarassunta; **Renzini Alessandra**; Mangialardo, Claudia; Cammarata, Ilenia; Santaguida, Maria Giulia; Virili, Camilla; Centanni, Marco; Adamo, Sergio; Moresi, Viviana; Verga-Falzacappa, Cecilia. Int J Mol Sci. 2019 Nov 15;20(22). pii: E5754. doi: 10.3390/ijms20225754 (IF 4.6).

**5)** Interplay between Metabolites and the Epigenome in Regulating Embryonic and Adult Stem Cell Potency and Maintenance.
Harvey A, Caretti G, Moresi V**, Renzini A**, Adamo S. 2019.
Stem Cell Reports. Oct 8;13(4):573-589.
doi: 0.1016/j.stemcr.2019.09.003 (IF 5.5).

**6)** HDAC4 Regulates Skeletal Muscle Regeneration via Soluble Factors.
**Renzini A**., Marroncelli N., Noviello C., Moresi V., Adamo S. Front Physiol. 2018 Sep 27;9:1387 (IF 4.1).

**7)** Culture conditions influence satellite cell activation and survival of single myofibers.
**Renzini A**., Benedetti A., Bouchè M., Silvestroni L., Adamo S., Moresi V. Eur J Transl Myol. 2018 May 29;28(2):7567.

**8)** HDAC4 preserves skeletal muscle structure following long-term denervation by mediating distinct cellular responses.
Pigna E., **Renzini A.,** Greco E., Simonazzi E., Fulle S., Mancinelli R., Moresi V., Adamo S. Skelet Muscle. 2018 Feb 24;8(1):6 (IF 4.2).

**9)** Coordinated Actions of MicroRNAs with other Epigenetic Factors Regulate Skeletal Muscle Development and Adaptation.
Bianchi M., **Renzini A**., Moresi V. and Adamo S. International Journal of Molecular Sciences. 2017 Vol. 18, p. 1-14 (IF 4.6).

***CONFERENCE PRESENTATIONS***

*HDAC4 is important for preserving skeletal muscle structure and function in muscular dystrophy* **Alessandra Renzini**, S. Di Francescantonio, N. Marroncelli, M. Bianchi, S. Adamo, V. Moresi.
(Oral Presentation) 8th MyoGrad Summer School for Myology Paris │Berlin │Versailles 6 - 10 June 2017, Paris (FR).

*HDAC4 mediates multiple cellular responses in skeletal muscle*
**Alessandra Renzini**, Eva Pigna, Nicoletta Marroncelli, Sergio Adamo and Viviana Moresi.
(Poster Presentation) International conference on muscle wasting, molecular mechanisms of muscle wasting during aging and disease 23-28 Sep. 2018, Ascona (Switzerland).

*HDAC4 regulates skeletal muscle regeneration via soluble factors*
**Alessandra Renzini**, Nicoletta Marroncelli, Chiara Noviello, Viviana Moresi and Sergio Adamo
(Oral Presentation) IIM Myology Meeting 11-14 Oct. 2018, Assisi (PG).

*HDAC4 preserves skeletal muscle structure and function in muscular dystrophy*
**Alessandra Renzini**, Sergio Adamo and Viviana Moresi
(Poster presentation) International conference Parent Project. Feb. 15-17 2019, Rome, Italy

*HDAC4 preserves skeletal muscle structure and function in muscular dystrophy*
**Alessandra Renzini**, Sergio Adamo and Viviana Moresi
(Poster presentation) Myogenesis Gordon Research Conference: Building, Maintaining and Regenerating Skeletal Muscle. June 9-14, 2019, Lucca (Barga), Italy.

*New emerging role for HDAC4 on sarcolemma stability in DMD*
**Alessandra Renzini**, Sergio Adamo and Viviana Moresi
(Oral Presentation) IIM Myology Meeting 17-20 Oct. 2019, Assisi (PG).

*Culture conditions and muscle morphology: an ex vivo analysis on single myofiber*
**Alessandra Renzini**, Anna Benedetti, Marina Bouchè, Leopoldo Silvestroni, Dario Coletti, Sergio Adamo and Viviana Moresi
(Oral Presentation) Morfologia e dintorni 3° incontro nazionale 26.9. 2020 (Online)

## FUNDINGS

"Avvio alla ricerca" Sapienza (2020)
"Avvio alla ricerca" Sapienza (2019)

## *Courses/Seminars* 2019/2020

- Le Basi Neuropsicologiche Dei Bias Cognitivi Accademia Medica Di Roma 05/03/2020 (online)
- "La percezione pubblica della scienza: i giovani ricercatori di fronte a temi scientifici di forte interesse pubblico, politico e mediatico" F.to Prof.ssa Elena Cattaneo F.to Prof. Marco Muzi Falconi 27-28/04/2020 (online)
- From constitutive heterochromatin to chromatin remodeling and human developmental diseases: the short story of a long trip Patrizio Dimitri 20/05/2020
- "Covid-19: come si insegue e sconfigge un virus" 28-29/05/2020 Dipartimento di Bioscienze dell'Università di Milano e dal Centro UniStem (Online)
-"Pandemia da SARS-CoV-2: Aspetti Virali, Epidemiologici, Clinici, Immunologici, e Terapeutici" Accademia Medica Di Roma 04/06/2020 (online)
- On-Line PATHBIO Anatomy course 6-17/07/2020 (78 hours duration)
- Virtual FISV Symposium on SARS-CoV-2 Biology and COVID-19 Current research and perspectives 16/09/2020 (Online)
- Formazione trasversale dottorandi (online):
    Europrogettazione (progetti collaborativi) in ambito di Programmi di Ricerca & Innovazione. Scouting di finanziamenti tramite l'utilizzo di Research Professional 22/09
    Open Access delle pubblicazioni e dei dati della ricerca 23/09
    Catalogo delle pubblicazioni IRIS: finalità e modalità 23/09

- Morfologia e dintorni 3° incontro nazionale 26.9. 2020 (Online)
- BeMM:

EPIDEMIOLOGICAL AND MATHEMATICAL MODELLING OF SARS-CoV-2 INFECTION
Thursday, September 17, 2020
CLINICAL AND IMMUNOLOGICAL FEATURES OF COVID-19
Thursday, September 24, 2020 8.30 am
COVID-19 LEGAL ISSUES
Thursday, October 1, 2020
THE IMPACT OF THE COVID-19 PANDEMIC ON HUMAN RIGHTS
Thursday, October 8, 2020
(online)

**MIT PhD Student seminars:**

12/12/2019 Bone cell mechanotransduction: from mechanical stimulus to cell elasticity measurement Ludovica Apa
12/02/2020 Dissecting the interplay between endothelin receptor and B1 integrin in invadopodia function and metastatic process of ovarian cancer Ilenia Masi
12/02/2020 Modellizzazione neurofisiologica dello stato cognitivo di lavoratori europei in età avanzata Vincenzo Ronca
19/02/2020 Ruolo degli RNA non codificanti nel controllo post-trascrizionale dell'espressione genica nel tumore alla mammella triplo negativo Gabriella Esposito
19/02/2020 Dissecting the role of novel onco miRs and their molecular targets in the evolution of resistence to target therapies in melanoma Vittorio Castaldo
26/02/2020 Role of N6-methyladenosine in skeletal muscle regeneration Francesco Millozzi
26/02/2020 Collagen architecture and stiffness as biophysical constraints in the modulation of epithelial-mesenchymal transition Noemi Monti

04/03/2020 Retinoic acid, proteasome inhibition and oxidative stress as a new combined strategy to induce myeloid leukemic progenitor cell death Francesca Liccardo
04/03/2020 Role of circRNAs-m6A dependent during stress response in myeloid leukemic cells Alessia Iaiza
22/04/2020 Lab-on-chip systems for the detection of pathogens Manasa Nandimandalam
28/04/2020 The effects of extracellular vescicles on ALS ex-vivo and in-vivo models: development of innovative functional measurement techniques and testing tools Flavia Forconi
06/05/2020 The cellular mechanotransduction: from mechanical stimuli to cellular young's modulus measurement Serena Carraro

06/05/2020 Gene therapy for Ataxia-Telangiectasia syndrome Bruna Sabino
13/05/2020 Control of macrophage programming in the tumor microenvironment: Interplay between TPC2/calcium signaling and autophagy Samantha Barbonari
13/05/2020 Toxicogenomic effects of Benzoapyrene on human adult testis Irene Tartarelli
20/05/2020 From constitutive heterochromatin to chromatin remodeling and human developmental diseases: the short story of a long trip Patrizio Dimitri
22/05/2020 Non invasive analysis of the embryonic genome for the development of improved preimplantation genetic testing protocol Antonio Capalbo
22/05/2020 Molecular imaging gamma tomosynthesis for small lesion detection in nuclear medicine examinations Maria Concetta Longo
27/05/2020 Theranostic applications of Graphene Oxide Riccardo Di Santo
27/05/2020 Cytoplasmic HDAC4: is it simply an inhibited epigenetic factor in DMD? Alessandra Renzini
03/06/2020 Kinematic study on ALS Marco Ceccanti

03/06/2020 The activation of miR-125a-5p/IP6K1 axis upon myo-Inositol administration: a potential novel target for breast cancer therapy Mirko Minini
10/06/2020 A novel 3D culture system as an in vitro model to study muscle biopsy an disease Marianna Cosentino
10/06/2020 Hormonal involvement and new drugs delivery system against melanoma disease Giada Pontecorvi
17/06/2020 Role of DACH1 in prostate cancer and its involvement in radioresistance Ilenia Giordani
17/06/2020 Molecular and cellular networks driving neurogenic muscle atrophy Daisy Proietti
24/06/2020 Anti-tumor effect of oleic acid in hepatocellular carcinoma cell lines via autophagy reduction Federico Giulitti

24/06/2020 Role of muscarinic receptors in epithelial ovarian carcinoma Marilena Taggi
01/07/2020 Analysis of phosphodiesterases expression during heart development and role of PDE5 in neonatal cardiomyopathy induced by gestational diabetes Thays Maria Carvalho
01/07/2020 Molecular characterization of ALS pathogenic mechanisms Elisa Lepore
08/07/2020 Oxytocin as physiological anticachetic agent Alexandra Benoni
08/07/2020 Role of STAT3 signaling in control of muscle regeneration, growth and autophagy during age-related degenerative neuromuscular diseases Giorgia Catarinella
15/07/2020 The interplay between metabolic alterations and circadian clock Irene Casola
15/07/2020 The interplay between bARs signalijng and clock genes in cardiac tissue pathophysiology Elena Crecca
22/07/2020 Characterization of non-human primate spermatogonial compartment Chiara Capponi
22/07/2020 Analysis of radioresistence mechanisms in a model of bone metastasis from prostate cancer Silvia Sideri

29/07/2020 C-Met-activated signalling pathway promotes malignant behaviour of NT2D1 non-seminoma cell line Luisa Gesualdi
15/09/2020 Brain K-tomography Marco Bettiol
15/09/2020 Brain segmentation in preclinical and clinical MRI Riccardo De Feo
23/09/2020 Targeting PKC theta to ameliorate dystrophic heart fibrosis in a novel mdx model of accelerated pathology Jacopo Morroni
23/09/2020 Epigenetic role of nuclear microRNAs in normal and neoplastic hematopoiesis Martina Gentile
25/09/2020 Role of Phosphodiesterase type 5 in the induction of cardiac hypertrophy Ana Gabriela Rego de Oliveira
29/09/2020 X-ray phase contrast tomography applied to flagship medical problems Nicola Pieroni
29/09/2020 Keratin-coated gold nanoparticles functionalization with 99mTc-FDG for selective anticancer photothermal therapy Mariano Pontico
07/10/2020 Neurophysiological models to predict the mental state of users in industrial context Alessa Vozzi

## POINT BY POINT RESPONSE

We greatly appreciate both reviewers highlighting the strengths of my PhD thesis. We have discussed and incorporated all the changes recommended, as outlined below.

**Reviewer #1**

In this thesis, the PhD candidate Alessandra Renzini focused on studying the role of HDAC4 in Duchenne Muscular Dystrophy (DMD). In particular, by crossing mice in which HDAC4 is specifically deleted in myogenin-expressing cells with mdx (the murine model of DMD), Alessandra characterized the histological, functional and cellular events triggered by muscle specific HDAC4 deletion in dystrophic muscles. By using a number of histological and functional assays, the candidate convincingly shows that deletion of HDAC4 worsens the dystrophic phenotype. Despite no differences in the caliber of myofibers observed between mdx and mdx;HDAC4cKO muscles, Dr. Renzini shows several evidences that support increase degeneration, and decreased regeneration, occurring in dystrophic muscles depleted of HDAC4. From a mechanistic point of view, Dr Renzini explored the possibility that lack of HDAC4 might worsen degeneration of mdx muscles affecting the membrane repair pathway. This last part, although testing an intriguing hypothesis, is based on correlative evidences and the molecular mechanism by which HDAC4 can regulate Trim72 or Dysferlin remains elusive. As also discussed by the candidate, it is not clear whether lack of HDAC4 is directly responsible of Trim72 expression and/or localization. Overall, the thesis is well written, and the candidate comprehensively introduced all the relevant arguments needed to fully understand and evaluate the experimental section, showing a solid knowledge of the literature in the field. I am not used to thesis combining Results and Discussion sections, but I assume

this format is allowed and the candidate well discusses possible caveats or interpretations of her results (with some points listed below).

Thanks to the reviewer for appreciating my work.

Here is a list of few points/suggestions that can be further discussed/modified in the final version of the work:

- it is not clear if defects in satellite cells are cell-autonomous (as fig.7 would suggest) or caused by the lack of paracrine factors due to the loss of HDAC4 (as implied in Fig.10). It seems from the data that it might be both, but it is not very clear how. Being HDAC4 knocked in myogenin expressing cells I would say that defects observed in SCs should be mostly cell-autonomous. However, a lot of emphasis is put on the paracrine effects either from myofibers (or FAPs). This in my opinion might be due to "only" secondary effects caused by more degeneration observed in HDAC4-depleted muscles (as compared to the mdx). The candidate should discuss this aspect and, although she cites previous work, she could speculate on which of these factors are thought to mediate this function in this model.

In figure 7, the intrinsic myogenic potential of mdxHDAC4mKO satellite cells (SCs) has been shown: primary SCs were isolated from mdx and mdx;HDAC4mKO mice and cultured in vitro under standard conditions to assess their intrinsic ability to differentiate and fuse in myotubes. From this analysis, we concluded that mdx;HDAC4mKO SCs showed compromised differentiation, fusion and increased SC death in vitro, differently from HDAC4mKO SCs. This phenotype is likely due to a cell-autonomous function of HDAC4, since it is deleted during SC differentiation, as the reviewer pointed out.

We then wondered whether HDAC4 mediates the release of soluble factors in the dystrophic context, as it does upon chronic injury, affecting SCs differentiation. To address this point, we evaluated the effects of mdx

and mdx;HDAC4mKO mice-derived serum on survival and myogenic ability of healthy SCs, confirming that HDAC4 mediates the released of soluble factors that influence SCs in mdx context.

Considering that i) serum is a source of a multitude soluble factors resulting from several cells and tissues; ii) HDAC4 is deleted in myogenin expressing cells, as indicated by the reviewer and iii) we observed an affected FAPs behavior in mdxHDAC4mKO mice, we wondered whether HDAC4 from muscle or impaired muscle niche/environment in vivo directly affects FAPs biology. On these bases, we performed mdx FAPs cultures in vitro, by using conditioned media derived by mdx or mdx;HDAC4mKO single myofibers. In these experiments, isolated myofibers do not show any sign of muscle necrosis *in vitro*. Shown results (Figure 14) confirmed mdx;HDAC4mKO myofibers as a direct source of soluble factors affecting mdx FAPs, but not mdx SCs (data not shown). Moreover, co-culture experiments with mdx SCs and either mdx or mdx;HDAC4mKO FAPs indicate that affected mdx;HDAC4mKO FAPs, in turn, modulate mdx SC behavior.

I agree with the reviewer defining effects on FAPs as “secondary” due to HDAC4 depletion in skeletal muscle; however we clarify that FAPs biology directly depends on soluble factors released (or not) by HDAC4 from myofibers and not by other cells (i.e. inflammatory cells) widely present when muscle degeneration occurs. Overall, we speculate that HDAC4 plays multiple functions in dystrophic muscles, by affecting SCs in cell-autonomous manner and by releasing soluble factors that influence both mdx FAP and SC behavior.

Based on your comment, I have extended the discussion of our data in the specific section of my thesis (pages 46-47) as reported below.

...”Overall, we speculate that HDAC4 plays multiple functions in dystrophic muscles, by affecting SCs in cell-autonomous manner and by

releasing soluble factors that influence both mdx FAP and SC behavior. In addition to regulate mdx;HDAC4mKO SC differentiation and survival, HDAC4 regulates the release of soluble factors from myofibers able to affects FAPs biology, as shown by conditioned-medium experiments *in vitro*. Moreover, inflammatory cell composition seems to be unaffected in mdx;HDAC4mKO mice, excluding a possible secondary effect on FAPs due to a widespread muscle degeneration. Myofibers are source of myokines and soluble molecules able to affect several cell types, including FAPs. For instance, upon muscle injury, myofibers produced IL-15 able to inhibit adipogenesis of FAPs[11]. In addition, skeletal muscle is known to produce extracellular vesicles (EVs) in response to different stimuli (i.e. exercise and pathologies), thereby affecting muscle environment [12,13]. Our results indicate HDAC4 involvement to modulate soluble factors or EVs content from myofibers which in turn affect FAPs behavior in mdx mice. Importantly, in vitro conditioned-medium experiments demonstrated that HDAC4 from skeletal muscle does not directly influence mdx SCs behavior but needs FAPs as cellular mediators for exerting its function. Further on going- experiments are aimed to characterize mdx and mdx;HDAC4mKO myofiber-secretome, in order to identify the soluble factors able to affect FAPs biology.

Recently, an important role for HDACs in affecting FAP-derived EV content in DMD has been reported[6]. Exposure of mdx FAPs to pan-HDACi increases the expression and the release of miR-206 in EVs, favoring muscle regeneration and reducing fibrosis and inflammation in mdx mice. Our results demonstrate a crucial role for HDAC4 in skeletal muscle: if deleted, mdx FAPs showed a more pro-adipogenic phenotype and decreased ability to promote mdx SC differentiation. Our results are important considering that pan-HDACi are systemically administrated in DMD patients. In the future experiments, we plan to analyze FAP EV content in our experimental model."

- in light of the increased adipogenic differentiation observed in FAPs from mdxHDAC4cKO, I wonder if these muscles also show increased adipogenic infiltration. If these data are available, they could be included. Or at least this could be discussed.

Adipogenic infiltration was not yet evaluated in our mouse model. Thank to your comment I started analyzing this relevant point. I evaluated adipogenic infiltration in old (16 months-old) mdx;HDAC4mKO and mdx GA muscles by Oil Red O (ORO) staining (new Fig 13). Quantification of ORO-positive area indicates increased adipogenic content in mdx;HDAC4mKO muscles compared to mdx ones. Based on *in vitro* FAPs experiments, we expect to observe higher adipogenic infiltration also in mdx;HDAC4mKO young (6 weeks-old) mice respect to mdx ones. We plan to investigate on fibro-adipose muscle infiltration *in vivo* at different time points to further confirm HDAC4 role in maintaining muscle homeostasis in DMD.

I have extended the results and discussion of these *in vivo* data in the revised thesis (pages 42-43) as reported below.

"FAPs are the mainly source of fat deposition in aged mdx muscles[215], therefore we wondered whether mdx;HDAC4mKO muscles show increased adipogenic infiltration *in vivo*. Fat deposition was analyzed by ORO staining in 16 month–old mdx;HDAC4mKO and mdx GA muscles. A significant increase of adipogenic content in mdx;HDAC4mKO GA muscles compared to mdx ones was quantified (Fig. 13).

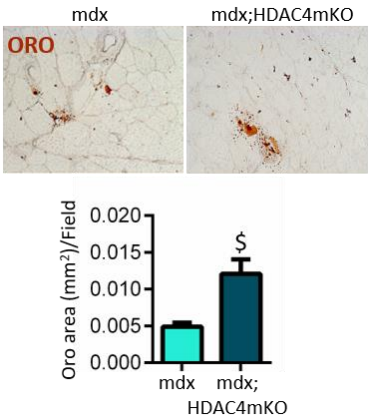



***Figure 13. Deletion of HDAC4 in mdx skeletal muscles exacerbates fat deposition***

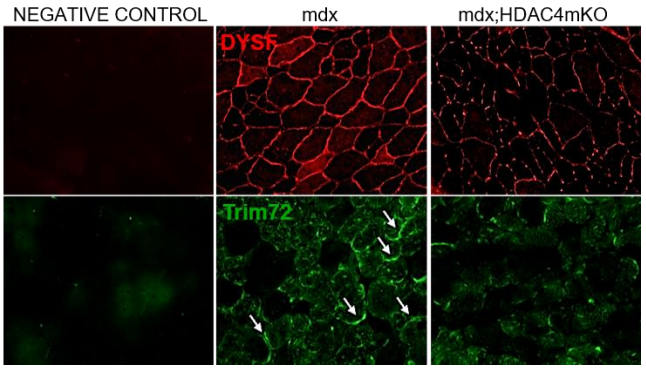
Representative images of mdx and mdx;HDAC4mKO GA muscles at 16 months stained with ORO and quantification of fat accumulation in muscles. Data are expressed as mean +/− SEM. n=3 mice per each genotype; $p<0.001 by Student's t test.

The pro-adipogenic commitment of FAPs is influenced by several pathways in dystrophic muscles. Inflammatory environment and myofiber-released signals cooperate to inhibit FAPs adipogenesis in young mdx muscle avoiding fat deposition. Differently, decreased inflammation in old mdx mice remove important signals to control FAPs behavior, which strictly depends by muscle signaling at this stage[194]. Our result indicate that fat deposition is exacerbated in mdx;HDAC4mKO mice compared to mdx ones, thus confirming HDAC4 signaling crucial to modulate FAPs behavior in dystrophic context."

- Immunofluorescence for Dysferlin and Trim72 in Fig.17b is not very convincing. Skeletal muscle is highly autofluorescent in green and it is hard to appreciate if this is a real signal or just background. Negative controls (i.e. the background of only secondary antibodies) should be shown. As stated in the introduction, Dysferlin and Trim72 should be

localized in the sarcolemma and this is not clearly appreciable by the shown images, especially for Trim72. I understand that these pictures are from mdx mice, but is the localization affected by the absence of dystrophin? Showing the same IF also on wt muscles might be another way to strengthen the reliability of the staining. Otherwise, it is hard to conclude that lack of HDAC4 mislocalize Dysf and Trim72 in dystrophic muscles and this statement should be avoided.

We included the background of only secondary antibodies in the revised figure 17b (new 18b, page 50).




-In Fig. 20b, 25b and 31, why GFP IF is shown to evaluate the differentiation potential? It would be better to show MyHC staining.

GFP was used to evaluate transfection efficiency and fusion ability, not differentiation potential in Fig. 20b,25b, and 31. Fusion index and satellite cell survival were quantified in these transfection experiments as read out of satellite cell potential. We could not evaluate MyHC+ cells (differentiation index) since in some experiments c-myc-tagged proteins were used and both c-myc and MyHC primary antibodies derived from mouse.
Based on your advice and to enrich our analysis, we plan to perform MyHC staining whenever is possible in the transfection experiments.

- In Fig25, why the wt form of HDAC4 does not rescue the phenotype? This should be at least discussed.

Transfection with Lipofectamine needs to be performed in proliferating cells and HDAC4 is expressed and localize within the nucleus in proliferating myoblasts; therefore, we are overexpressing HDAC4 since the proliferation stage until differentiation. Our results are in line with previous data shown in literature. Indeed, wt HDAC4 as well as the mutant nuclear HDAC4 overexpression inhibits myoblast differentiation in vitro and exert deleterious effects on skeletal muscle in vivo[237, 238]. HDAC4 in proliferating myoblasts associates with and inhibits MEF2-dependent transcription, thereby inhibiting myogenesis. Upon differentiation, class II HDACs translocate into the cytoplasm, thereby releasing the inhibition on MEF2, which promotes myoblast differentiation.
Shedding light on HDAC4 cytoplasmic functions will provide a strategy to preserve its protective function without affecting HDAC4 nuclear levels, important for proper myogenesis.

As suggested, I have further discussed this point in the text (page 63), as reported below.
…"Our results are in line with previous data shown in literature. Indeed, wt HDAC4, as well as the mutant nuclear HDAC4 overexpression, inhibits myoblast differentiation in vitro and exert deleterious effects on skeletal muscle in vivo [14,15]. Indeed, HDAC4 localizes within the nucleus in proliferating myoblasts, where it associates with and inhibits MEF2-dependent transcription, thereby inhibiting myogenesis. Upon differentiation, class II HDACs translocate into the cytoplasm, thereby releasing the inhibition on MEF2, which promotes myoblast differentiation.
Overall, the transfection experiments clearly indicate that HDAC4 exerts

its protective functions in the cytoplasm of dystrophic SCs. Shedding light on HDAC4 cytoplasmic functions will provide a strategy to preserve its protective function without affecting HDAC4 nuclear levels, important for proper myogenesis."

Presentation and clarity
[ ] None [ ] Poor [ ] Average [X] Good [ ] Excellent

Integration and coherence

[ ] None [ ] Poor [ ] Average [X] Good [ ] Excellent

Introduction to scientific background

[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Review of relevant literature

[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent
Statement of research problem

[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Originality

[ ] None [ ] Poor [ ] Average [X] Good [ ] Excellent

Contribution to knowledge and scientific relevance

[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Mastery of the English language
[ ] None [ ] Poor [ ] Average [X] Good [ ] Excellent

A major goal of the review process is to evaluate if the present version of the thesis is:

[ ] Accept as is [X] Minor revision [ ] Major revision

Reviewer #2

The thesis is well written, pleasant to read and extremely focused on the subject of the research presented. I very appreciated the clearness of the

manuscript, which results very well balanced in the different parts in which it is correctly subdivided. The rationale, the experimental plan and the methods used are extremely appropriate. The work of this thesis represents the sequence of published results in the international journal Front Physiol., in which Dr. Alessandra Renzini appear as first author. I would like to underlay that a very great job has been done by Dr. Alessandra Renzini contributing to the generation and detailed characterization of dystrophic mice with a skeletal muscle-specific deletion of HDAC4. Several unpublished, original and interesting data are included in the thesis, including the identification of the specific role and the mechanisms of cytoplasmatic HDAC4 in preserving muscle homeostasis in the DMD context. The bibliography section is exhaustive, updated and appropriate. The work of Dr. Renzini represents an important advance in the field of DMD, offering alternative and future therapeutic approaches to preserve dystrophic muscle to damage. Thus, my evaluation about Dr. Renzini's thesis is extremely positive, being the thesis an outstanding work.

Thanks to reviewer for the supportive and encouraging positive feedback.

Presentation and clarity
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Integration and coherence
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Introduction to scientific background
[ ] None [ ] Poor [ ] Average [X] Good [ ] Excellent

Review of relevant literature
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Statement of research problem
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Originality

[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Contribution to knowledge and scientific relevance
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

Mastery of the English language
[ ] None [ ] Poor [ ] Average [ ] Good [X] Excellent

A major goal of the review process is to evaluate if the present version of the thesis is:
for admission of the candidate to the defense of the work in front of a national evaluation board.
[X] Accept as is [ ] Minor revision [ ] Major revision