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ORIGINAL PAPER

Faster regeneration associated to high expression of *Fam65b* and *Hdac6* in dysferlin-deficient mouse

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Abstract

Dysferlin is a sarcolemmal muscle protein associated with the processes of membrane repair, trafficking, and fusion of intracellular vesicles and muscle regeneration. Mutations in the *DYSF* gene cause clinically distinct forms of muscular dystrophies. The dysferlin-deficient *SJL/J* mouse model presents a reduction of 85% of the protein but shows mild weakness and discrete histopathological alterations. To study the effect of dysferlin deficiency in the muscle regenerative process, we used a model of electrical injury by electroporation to induce muscle degeneration/regeneration in the *SJL/J* mouse. The relative expression of the genes *Pax7*, *MyoD*, *Myf5,* and *Myog* was accompanied by the histopathological evaluation during muscle recovery at different time points after injury. We also investigated the effects of dysferlin deficiency in the expression of genes encoding FAM65B and HDAC6 proteins, recently described as forming a tricomplex with dysferlin at the beginning of myoblast differentiation. We observed an altered time course through the process of degeneration and regeneration in dysferlin-deficient mice, with remarkable regenerative capacity characterized by a faster and effective response in the first days after injury, as compared to the WT mice. Also, dysferlin deficiency seems to significantly alter the gene expression of *Fam65b* and *Hdac6* during regeneration, since higher levels of expression of both genes were observed in dysferlin-deficient mice. These results need further attention to define their relevance in the disease mechanism.

Keywords Limb-girdle muscular dystrophy 2B · Dysferlin · Myogenesis · Muscle regeneration

Introduction

Dysferlin is a ~ 230 kDa sarcolemmal protein expressed mainly in skeletal and cardiac muscle. Reduced levels or absence of dysferlin due to mutations in the *DYSF* gene cause a group of clinically heterogeneous myopathies characterized by progressive weakness and muscle degeneration, collectively known as dysferlinopathies. The different clinical presentations include Limb-girdle muscular dystrophy type 2B (Bashir et al. 1998; Vainzof et al. 2001), Miyoshi myopathy (MM) (Liu et al. 1998) and distal anterior compartment myopathy (DACM) (Illa et al. 2001).

Skeletal muscle is a mechanically active tissue undergoing frequent membrane stress, being, therefore, highly adapted to repairing when submitted to various stimuli or demands generated by physical activity, growth, and injury. However, in progressive conditions, such as muscular dystrophies, the imbalance between degeneration and regeneration cycles leads to degenerative processes and irreversible loss of the musculature with the advance of chronic disease (Ceco and McNally 2013). Understanding the mechanism of muscular disorders at cellular and molecular levels is essential for the development of therapeutic targets. Despite advances, the role of dysferlin still remains largely unclear. Some functions related to dysferlin in recent years include traffic and fusion of intracellular vesicles (Glover and Brown 2007), membrane repair (Bansal et al. 2003), inflammation (Rawat et al. 2010) and myogenesis (De Luna et al. 2006).

In dysferlinopathies, mechanical stress causes calcium imbalance and failure of membrane repair, which leads to proteolysis and prolonged exposure to an oxidative extracellular environment, conducting eventually to necrosis and

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disease progression (Kerr et al. 2014). Also, more recently, metabolic changes closely linked to high levels of lipids and toxicity were associated with this progressive disease in animal models for LGMD2B (Sellers et al. 2018). On the other hand, muscle necrosis and regeneration is not the main feature of dysferlinopathies histopathology, and loss of muscle mass and fat replacement is observed in late stages of the disease (Terril et al. 2013; Grounds et al. 2014).

The regenerative response to muscle degeneration is activated by the action of several myogenic determinants. In normal muscle, *Pax7* is involved in the activation of satellite cells committed to myoblasts formation of and muscle differentiation. Once activated, they give rise to precursors expressing a group of myogenic factors known as Myogenic Regulatory Factors (MRFs). Their expression is regulated temporally. *MyoD* and *Myf5* are the primary factors that act in the activation and proliferation of myogenic precursors cells, while *Myog* acts on terminal differentiation of myoblasts into myotubes (Rudnicki et al. 2008; Sabourin and Rudnicki 2000).

The observation that dysferlin and myogenin mRNA levels increase during myogenic differentiation of human skeletal muscle cells and that levels of myogenin decrease at myotube stages in dysferlin-deficient cells gave the first evidence that dysferlin may have important functions in myogenesis (De Luna et al. 2006).

Second evidence for this important role of dysferlin was suggested based on its described interaction with FAM65B and HDAC6, forming a transient tricomplex at the beginning of myoblast differentiation (Balasubramanian et al. 2014). Histone deacetylase 6 (HDAC6) is a cytoplasmic HDAC with an important role in regulating microtubule dynamics, controlling cellular motility, via deacetylation of several proteins, including α-tubulin. In differentiating muscle cells, dysferlin has been reported to bind HDAC6, thus preventing tubulin deacetylation (Di Fulvio et al. 2011). FAM65B is a cytoplasmic protein transiently upregulated during early myoblast differentiation and its expression is necessary for the binding between HDAC6 and dysferlin. When FAM65B expression is transiently elevated, HDAC6 activity decreases while the levels of acetylated tubulin and myogenin increase, promoting myotubes fusion (Balasubramanian et al. 2014). The effects of dysferlin deficiency in the tricomplex components and their expression during the regenerative process are still unknown.

Here we used *SJL/J* mouse, the natural murine model for LGMD2B, to study the regenerative process under dysferlin deficiency. In this model, a deletion of 171 bp in exon 43 of the dysferlin gene leads to a loss of 85% of protein levels in muscle. Although a good model for molecular studies, *SJL/J* displays a mild phenotype when compared to the phenotype in patients. Histological changes are also very mild, starting between 2 and 4 months of age, however, the active myopathy develops after 6 months (Weller et al. 1997). Thus, induction of muscle injury provides a quick and efficient mechanism to evaluate changes in the regenerative process of dystrophic animals with such mild phenotype. We used a technique developed in our laboratory to assess dystrophic muscle recovery after electrical-induced injury to study the influence of dysferlin deficiency in muscle regeneration in vivo. The degree and extent of injury were analyzed, as well as subsequent regeneration events by histological assessment, mRNA and protein analysis of myogenic factors and tricomplex components.

Our results suggest that there is no impairment of regeneration in dysferlin-deficient *SJL/J* mice. However, an altered time course of regeneration was observed, which was vigorous in the first days of muscle recovery in dysferlin-deficient mice, while less accentuated expression and prolonged myogenesis was observed in normal mice. Also, dysferlin deficiency seems to significantly alter gene expression of *Fam65b* and *Hdac6* during the time course of regeneration, since higher expression levels of both genes were observed in dysferlin-deficient mice. These results need further attention to define their relevance and application.

Materials and methods

Animals

We examined the gastrocnemius muscles of male *SJL/J* with 12–14 weeks of age and respective wild-type *Swiss* mice (WT). The outbred *Swiss* mouse was selected as normal control because of the same initial background of the two models since the *SJL/J* mice were developed in 1955 from three different sources of *Swiss* Webster mice (Jax Lab—https://www.jax.org/strain/000686)). *SJL/J* mice were obtained from our animal colonies and *Swiss* mice were obtained from the School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ/USP).

During the experiments, animals were bred and housed in controlled temperature and light in the animal house of Human Genome and Stem Cell Research Center (HUGCELL). All experiments were performed in accordance with the ethical committee of animal use of Institute of Bioscience of the University of São Paulo (protocol number 218/2015).

Muscle injury/repair system

We performed muscle electrical-induced injury by electroporation, standardized in our laboratory (Almeida and Vainzof 2019), that cause acute damage followed by rapid regeneration for the examination of histological and molecular changes after 3, 5, 10, 15, 21 and 30 days, compared

to uninjured muscles (day 0). A total of 42 mice for each strain was divided randomly at different time points (n = 6 per group).

The electrical pulses were applied in both legs of each animal in gastrocnemius region, under general anesthesia induced by intraperitoneal injection (ketamine 10% + xylazine 2% + acepromazine 1%, loading doses of 80, 10 and 3 mg/kg, respectively). The posterior legs were shaved and a conductive gel was used to improve the contact of the electrodes with the skin. Gastrocnemius was electrically injured by applying eight square electrical pulses of 100 V, 20 ms (milliseconds) per pulse at 0.5 s intervals through electrodes (7 mm diameter) using a BTX®-ECM®830 electroporator (Harvard Apparatus). With this methodology, the created lesion reaches about 10–20% of the muscle at day 3 and is expanded to about 50% of the crosssectional area at day 5, in a very similar pattern in all animals.

Tissue collection and histological analysis

We collected both gastrocnemius muscles, frozen in liquid nitrogen, to study gene expression and histological alterations at different time points during muscle recovery.

Hematoxylin–eosin (H&E): Transverse cross sections of right gastrocnemius were stained with H&E and histopathological evaluation was performed by generating a qualitative assessment of the changes in the morphology of muscle fibers, necrosis, the presence of centrally nucleated muscle fibers (CNFs), inflammation and connective tissue infiltration. In addition, CNFs were counted under × 20 magnification (five fields, n = 3 animals/experimental group), and results were represented as the number of CNFs/total number of myofibers in each section. The stained sections were observed under a light microscope (Zeiss).

Acid phosphatase: For histochemical detection of phagocytosis, muscle sections were incubated for 1 h at 37 °C in a solution (pH 4.0–5.0) containing pararosaniline solution 0.8 ml, sodium nitrite solution 0.8 ml, naphthol-AS-BI-phosphate (10 mg/ml, diluted in *N*,*N* = dimethylformamide) and veronal acetate buffer (5 ml/13 ml of distilled H_2O), washed, stained with methyl green for 3 min, dehydrated in alcohol and mounted with Canada balsam. The acid phosphatase activity indicates active phagocytosis in degenerating events, usually in the acute phase, showing necrosis or response to mechanical stress or disease, which is marked with a red stain. A qualitative analysis, evaluating the intensity of positively labeled fibers was done (n = 6 animals/experimental group).

Picrosirius: To identify regions with collagen deposition, indicative of connective tissue replacement and infiltration between muscle fibers, the sections were stained with Sirius red (0.2 g) dissolved in saturated picric acid (100 ml) for 40 min. The slides were immersed in Bouin fixative for 20 min, washed in water, dehydrated and mounted in Canada balsam. The differences were quantified by the positive area of staining, labeled in red, relative to the total area of the section, with the aid of ImageJ software (3–4 fields, n = 5–6 animals/experimental group).

Immunofluorescence analysis for developmental isoform myosin heavy chain (dMHC): indicates myogenic activity by newly differentiated fibers labeled in red. The muscle sections were co-labeled with primary antibodies to dMHC (1:30; Vector; VP-M664) and laminin (1:50; Dako; Z0097), washed, and then incubated for 1 h with secondary antibodies Cy3-labeled sheep anti-mouse IgG (1:100; Sigma; C2181) and FITC-labeled donkey anti-rabbit IgG (1:100; Amersham; N1034), respectively. After washing, the slides were mounted with Vectashield-DAPI. The analysis of histological sections was done with a fluorescence microscope (Zeiss) and quantified the percentage of dMHC +/total fibers (5 fields, n = 3–6 animals/experimental group).

RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA from left gastrocnemius was extracted using the RNeasy Microarray Tissue Mini Kit (Qiagen), following manufacturer' s instructions and then quantified in a spectrophotometer. For the cDNA synthesis, 1 µg of total RNA was used with random primer 0.1 µl (3 µg/µl), 1 µl oligo dT (500 µg/ml), 1 µl dNTP mix (10 mM) and DEPC water adjusted to the volume of 12 µl. This mixture was left at 65 °C in a thermocycler for 5 min. Then was added 4 µl 5 × First Strand Buffer, 2 µl DTT (0.1 mM) and 1 µl RNase OUT (40 U/µl) to incubation in the thermocycler at 37 °C for 2 min. After removing the samples 1 µl of MMLV reverse transcriptase was added and samples were then incubated at 25 °C for 10 min, 37 °C for 50 min and finally at 7 °C for 15 min. The cDNA was diluted (1:3 in RNase free water) and applied in duplicates in 96 well plates. Samples were amplified using the MasterMix containing Sybr Green (Applied Biosystems) and primers (detailed in Table 1) in a total volume of 20 µL. Each plate was run on thermocycler 7500 Fast (Applied Biosystems/Life). To data analysis, each time point was normalized by Gapdh mRNA and the uninjured WT mice and expressed as 2-ΔΔCT.

Western blotting

Gastrocnemius muscle extracts were prepared in RIPA buffer (Sigma-Aldrich) and protease and phosphatase inhibitors (Sigma-Aldrich) and quantified using BCA™ Protein Assay Kit (Pierce). Equal amounts of protein (30 ug) were resolved by 13% SDS gels or 4–15% gradient Mini-PROTEAN® TGX™ Precast gels (Bio-Rad) and transferred onto nitrocellulose membrane using iBlot® Gel Transfer Device (Invitrogen). The membranes were blocked in 5% milk and

Table 1 Primers sequences

Gene	Forward sequence	Reverse sequence
Pax7	GAGTTCGATTAGCCGAGTGC	GAGTTCGATTAGCCGAGTGC
MyoD	TACAGTGGCGACTCAGATGC	TAGTAGGCGGTGTCGTAGCC
Myf5	CTGTCTGGTCCCGAAAGAAC	GACGTGATCCGATCCACAATG
Myogenin	CAGTACATTGAGCGCCTACAG	GGACCGAACTCCAGTGCAT
Fam65b	GGGCTTGATGAGTACCTGGA	GAAGGCTTGCTTCATTTTGC
Hdac6	AAGTGGAAGAAGCCGTGC TA	CTCCAGGTGACACATGATGC
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

incubated with primary antibodies for myogenin (1:250; BD; 556358), dysferlin (1:50; Vector; VP-D503), FAM65B (1:1000; Abnova; 2E6-1B10), HDAC6 (1:200; Santa Cruz; sc-11420) and GAPDH (1:1000; Cell Signaling; 2118), as internal control, overnight at 4 °C, followed by incubation with HRP-conjugated secondary antibodies. The immunodetection was visualized by ECL detection (Invitrogen) with Image Quant LAS 4000 Mini System (GE Healthcare). Membranes were stripped with Restore™ Western Blot Stripping Buffer (Thermo Scientific), when necessary. The protein band intensities were analyzed by ImageQuant TL 8.1 Software (GE Healthcare), normalized to GAPDH expression and expressed as the relative mean intensity of the uninjured WT muscles.

Statistical analysis

Differences between groups were assessed using Kruskal–Wallis followed by Dunn-Bonferroni test and Mann–Whitney when comparing only two groups. The statistical analyses were calculated using Minitab and a p value of less than 0.05 was considered to be significant. Asterisks indicate differences relative to the uninjured controls within each strain and # indicates differences between strains.

Results

Altered time course of regeneration in dysferlin-deficient mice

Morphological changes were evaluated during the regeneration process at different time points after electrical-induced injury. The uninjured muscle of *SJL/J* had discrete dystrophic alterations, with sparsely centrally nucleated muscle fibers. Histological changes observed in both strains after injury included foci of muscle degeneration and infiltration of mononuclear cells, mainly in the first days after injury. Intense inflammation and necrosis with hyaline ghost-like appearance were observed at day 3 and 5. From day 10, they start to decrease. At day 15, there is extensive regeneration in *SJL/J* muscle, however small degeneration associated to some degenerating foci are still observed near the periphery in WT mice, indicating a faster muscle recovery in dysferlin-deficient mice (Fig. 1a).

Signs of regeneration appeared at day 3 and became more evident at day 5 with small basophilic fibers. In addition, the quantitative analysis revealed that the number of centronucleated fibers (CNFs) increased from day 3 to 5 in both strains, and remained elevated, as observed in the murine regenerated muscle (Fig. 1b).

Efficient restoration of fibrosis

To investigate the ability of dysferlin-deficient mice to restore muscle cytoarchitecture, the dynamic deposition and remodeling of the connective tissue were visualized by picrosirius red staining, identifying positively areas with collagen. Our data showed extensive areas of positive staining, mainly on day 3 and 5, with a peak at day 5 in both strains. The extent of connective tissue was reduced in the subsequent days and replaced by newly formed fibers, with no significant differences between the strains, except at day 15, in which WT mice appears increased compared to the *SJL/J,* indicating that connective tissue replacement is not the main feature in the dysferlin-deficient mouse (Fig. 2a). These observations were confirmed by quantitation of area of collagen deposition (Fig. 2b).

Early activation of myogenic markers in dysferlin-deficient mice

The regenerative response to injury was further studied by examining gene expression levels of myogenic transcripts *Pax7*, *Myf5*, *MyoD,* and *Myog*, as compared to dysferlin expression, to determinate whether dysferlin deficiency interfered in the earlier and later stages of muscle regeneration. All transcripts had increased expression mainly in days 3 and 5 after injury in both strains. However, an earlier stimulation of myogenesis was observed in *SJL/J*, which had peaked at day 3, decreasing at day 5, whereas normal mice had peaked only at day 5. From day 10 onward, mRNA levels decreased but appeared slightly increased in the WT mice compared to *SJL/J* until day 30 (Fig. 3a).

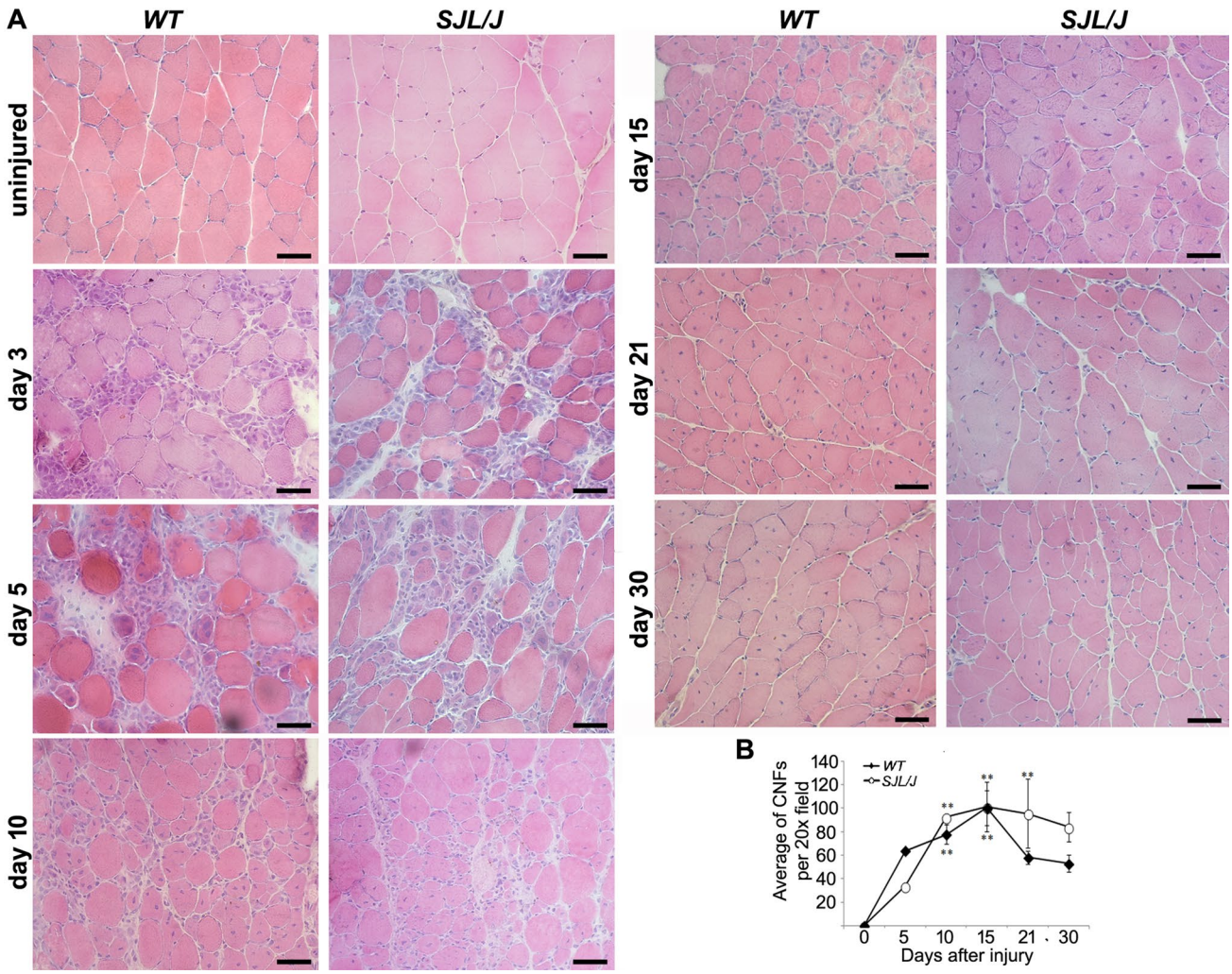

Fig. 1 Altered time course of muscle regeneration in dysferlin-deficient mice after electrical-induced muscle injury. **a** Gastrocnemius muscle from 3-month-old wild-type (WT) and *SJL/J* mice stained with H&E. **b** Average of CNFs (five fields, n = 3). Data are mean ± SD. Significant differences relative to uninjured muscle (day 0) (within each strain): *p < 0.05 and **p < 0.01. Bars: 50 µm

Comparing to WT mice, all myogenic transcripts were significantly higher in the dysferlin-deficient muscle on day 3. Then they appear similar at day 5, with the exception of *MyoD* that was significantly elevated in normal mice (Fig. 3b). Western blot analysis confirmed the previous findings, showing that electrical injury increased protein levels of myogenin in the first days after injury in both WT and *SJL/J* mice compared to uninjured muscle. A Significant higher level of protein myogenin was observed at day 3 after injury in *SJL/J* mice (Fig. 3c, d). These results suggest that early activation peak of myogenic factors could contribute to faster muscle regeneration observed in dysferlin-deficient *SJL/J*.

Based on these results, we then checked whether this profile is reflected in the pattern of regeneration of new myofibers using markers of developmental myosin isoform (Fig. 4a). During the first days after injury, fibers marked with a developmental isoform of myosin heavy chain (dMyHC) indicated newly formed fibers. Our data showed that the formation of new muscle fibers started on day 3, reached a significant peak at day 5 and decreased at day 10 in both strains (Fig. 4b). At day 5, the increased dMyHC positive fibers suggest no impairment of muscle recovery from injury in *SJL/J*, although no statistical significance was achieved between the strains (WT: 11.85% ± 4.89 vs. *SJL/J*: 18.78% ± 4.32; p = 0.06).

Dysferlin deficiency affects the components of the tricomplex

We next examined the time course changes in the expression of *Fam65b* and *Hdac6*, components of the tricomplex with dysferlin, to verify the pattern of expression during the different stages of muscle recovery and determine whether

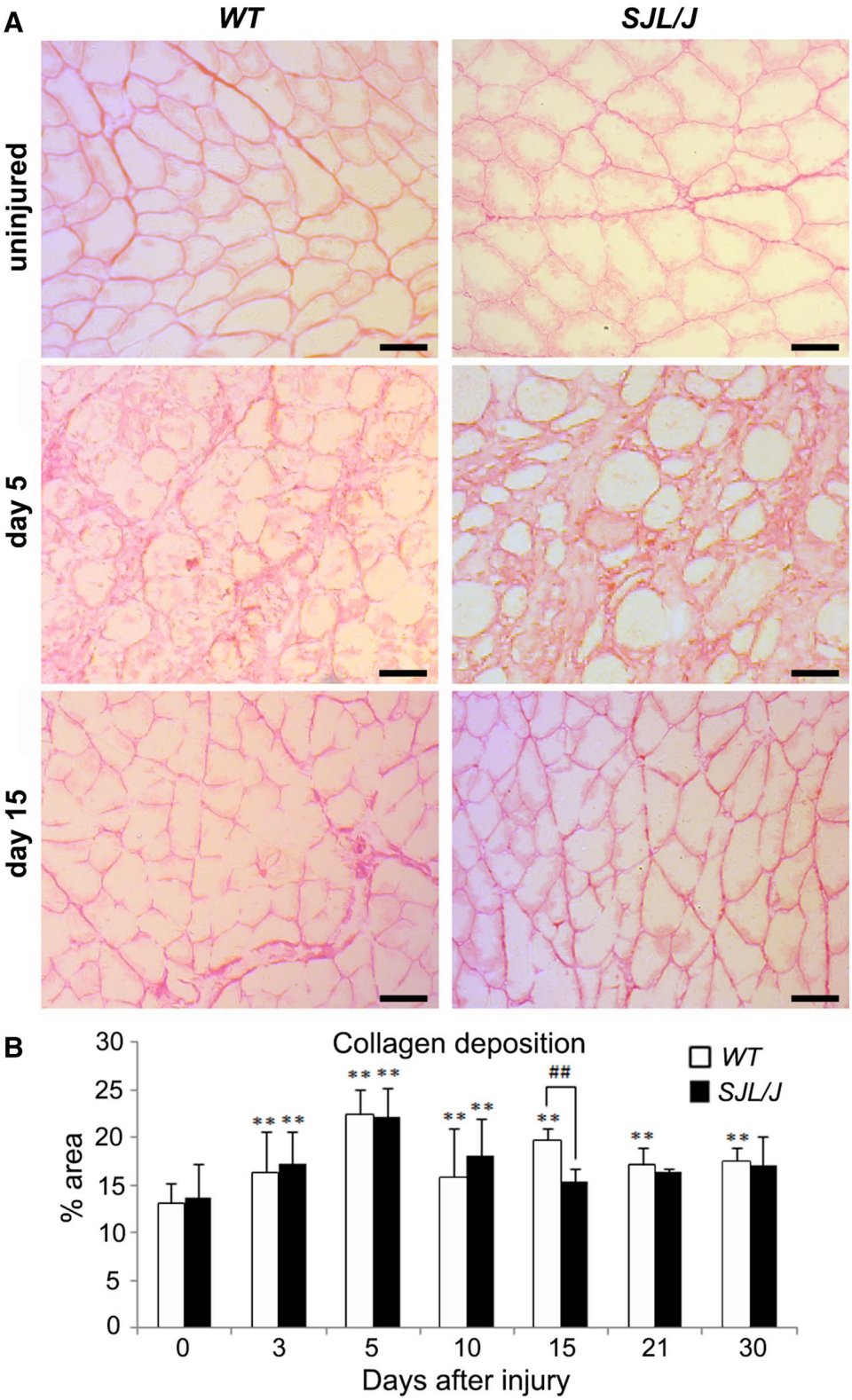

Fig. 2 Efficient restoration of collagen deposition. **a** Representative images of collagen deposition by Picrosirius red staining of gastrocnemius muscles of WT and *SJL/J* mice at day 3, 5 and 15 after electrical injury. **b** Quantitation of percentage area of collagen deposition (n = 3–6). Data are mean ± SD. Significant differences relative to uninjured muscle (day 0) (within each strain): *p < 0.05 and **p < 0.01 and between strains. ##p < 0.01. Bars: 50 µm

dysferlin deficiency interferes also in this pathway. *Fam65b* and *Hdac6* gene expression were upregulated during early regeneration, at days 3 and 5 in dysferlin-deficient and WT mice (Fig. 5a). However, in *SJL/J,* higher *Fam65b* mRNA levels were observed at day 3 and 5, and *Hdac6* at day 5, compared to WT mice (Fig. 5b). These results suggest that

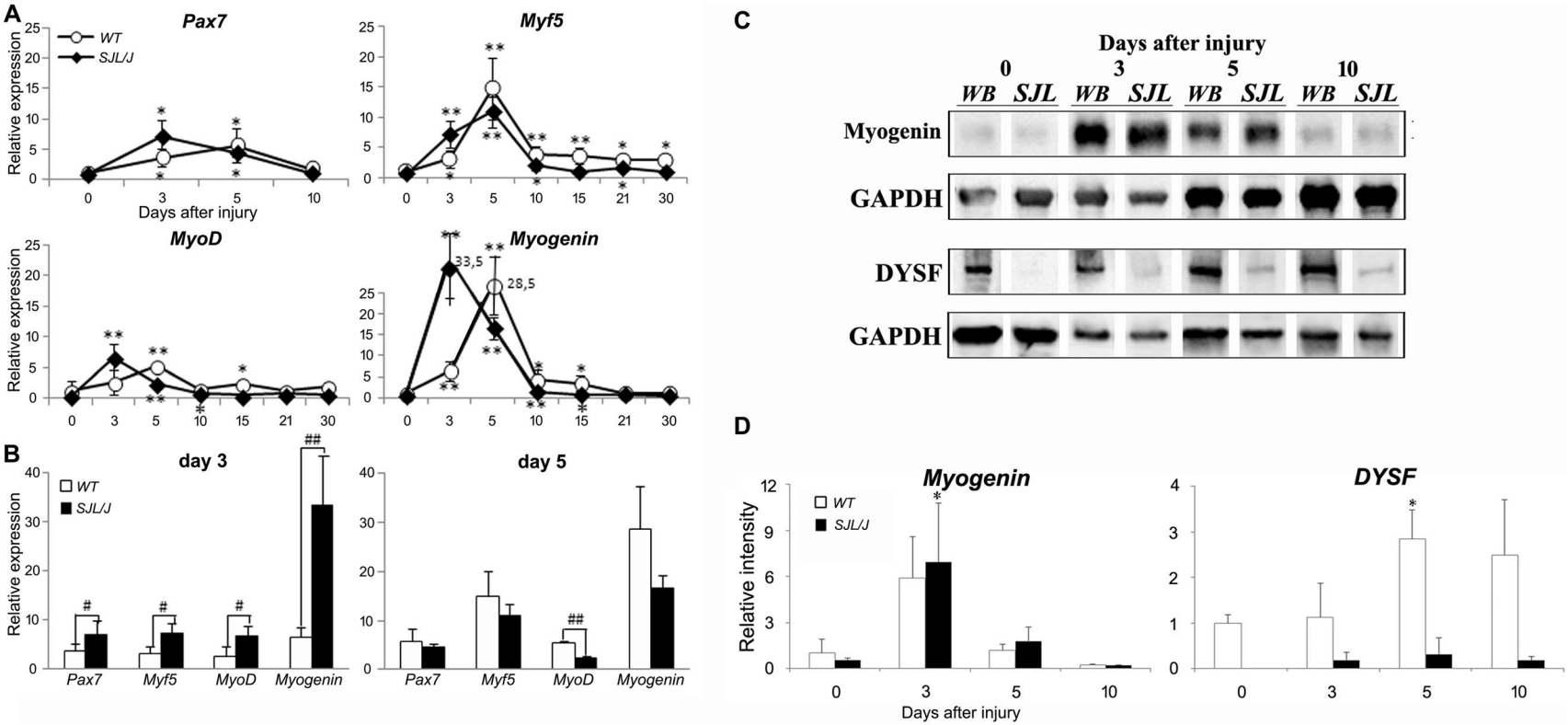

Fig. 3 Early activation of myogenic transcripts in dysferlin-deficient mice after electrical-induced muscle injury. **a** *Pax7*, *Myf5*, *MyoD* and myogenin mRNA levels in the injured muscles at different time points from WT and *SJL/J* gastrocnemius muscle determined by qRT-PCR. **b** Comparative analysis of all transcripts at day 3 and 5, highlighting the days of higher expression. Values are mean ± SD (n = 6). Significant differences relative to uninjured muscle (day 0) (within each strain): *p < 0.05; **p < 0.01, and between strains: $^{\#}p < 0.05$; $^{\#\#}p < 0.01$. **c** Western blot analysis for myogenin and dysferlin, and (D), protein quantifications

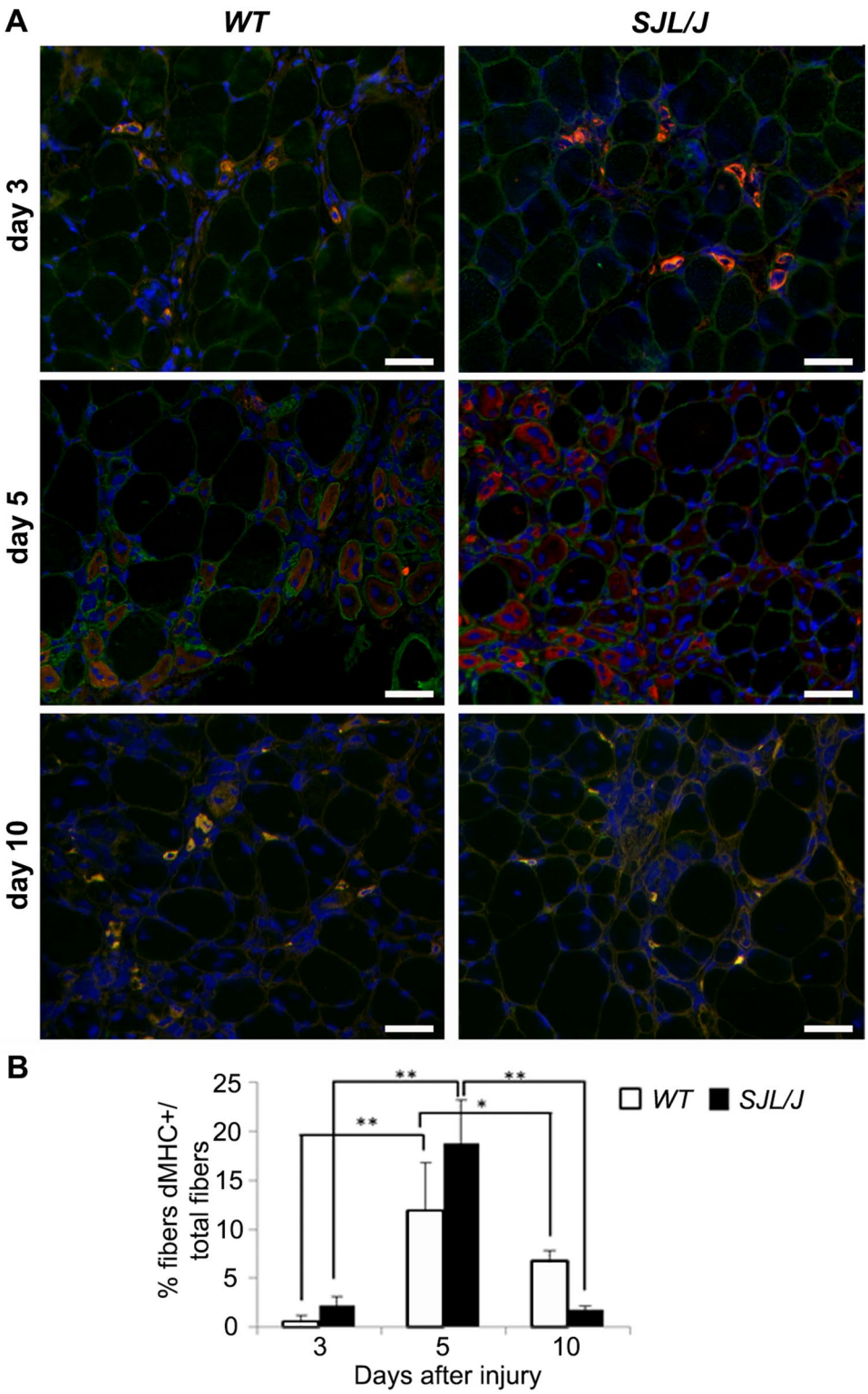

Fig. 4 *SJL/J* shows no impairment of new myofibers formation in the first days after injury. **a** Immunofluorescence staining for developmental isoform of myosin heavy chain (dMHC). dMHC + fibers are labeled in red to identify newly formed myofibers, indicating regenerative response after injury. Bars: 50 µm. **b** Quantitative analysis of percentage of fibers dMHC +/total fibers at days 3, 5 and 10 after injury. Values are mean ± SD (n = 3–6). Significant differences: *p < 0.05; **p < 0.01

Fam65b and *Hdac6* expression are affected by dysferlin deficiency.

Through Western blot analysis, the tricomplex components were also significantly elevated. FAM65B had peaked at day 5 and HDAC6 on day 3, in both strains. No differences were found at all time points between strains. As expected, the expression of dysferlin increased in WT in days 3 and 5, but not in *SJL/J* muscle (Fig. 3c, d).

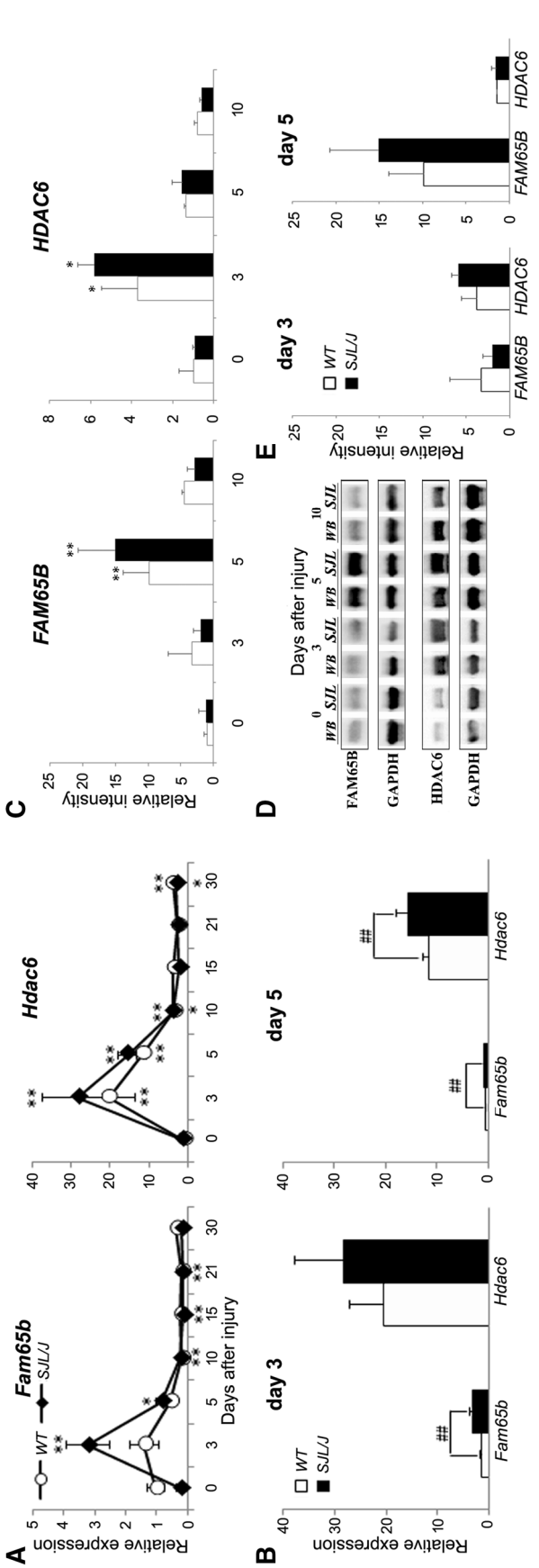

Fig. 5 Dysferlin deficiency affects the components of the tricomplex. **a** mRNA expression analysis of *Fam65b* and *Hdac6* in the injured muscle. **b** Comparative analysis of *Fam65b* and *Hdac6* at day 3 and 5. Values are mean ± SD (n = 6). Significant differences relative to uninjured muscle (day 0) (within each strain): *p < 0.05; **p < 0.01, and between strains: ##p < 0.01. **c** Quantitative analysis of protein band intensity at days 3, 5 and 10 after injury, relative to uninjured WT mice. **d** Representative western blot of FAM65B and HDAC6 protein levels from skeletal muscles at different time points post-injury in WT and *SJL/J* mice

Discussion

Many animal models for neuromuscular diseases are found in nature or produced in the laboratory. They can be useful for physiological, genetic and clinical studies. Such models mimic human diseases and thus can be used for better understanding of pathophysiological mechanisms, being powerful tools for the study of potential therapies (Vainzof et al. 2008). Here, we studied the regeneration of the dysferlin-deficient mouse *SJL/J*, a natural murine model for LGMD2B, largely used in studies of dysferlin deficiency. Due to the mild phenotype of disease observed in this model when compared to humans, we applied a methodology of electrical-induced injury developed in our laboratory (Almeida and Vainzof 2019). This method was chosen because it produces mechanical damage followed by a rapid regeneration, which allows us to analyze the events in a short period. Additionally, as compared to models of degeneration induced by some myotoxic and chemicals agents (Hardy et al. 2016), this mechanical method can be considered less interfering in the mechanisms of muscle degeneration and regeneration. The methodology was tested in normal mice (Almeida and Vainzof 2019), and compared with myotoxin induced lesions, with similar histological results (Almeida et al. manuscript in preparation).

Dysferlin is reported to increase during muscle differentiation, suggesting its role in regeneration and myoblasts fusion events (De Luna et al. 2006). Nevertheless, the underlying cellular mechanism of whether dysferlin acts in myotube formation remains unclear. Here, we assessed the histological and molecular events over the course of *SJL/J* muscle recovery after injury. The aim of this study was, therefore, to investigate how dysferlin deficiency affects the time course of degeneration/regeneration processes as well as how it modulates the expression of the genes encoding two recently described interacting proteins, FAM65B and HDAC6, that form a tricomplex with dysferlin (Balasubramanian et al. 2014). Our findings showed an altered time course of regeneration in dysferlin-deficient mice *SJL/J*, with intense inflammatory response, followed by efficient removal of necrotic tissue and faster muscle recovery compared to WT mice. We found that myogenic factors are upregulated earlier in this lineage. Moreover, the enhanced expression of *Fam65b* and *Hdac6* was observed. Taken together, active phagocytosis and early activation of myogenic factors appear to account for rapid regeneration in *SJL/J*.

Altered time course of regeneration in dysferlin-deficient mice

Despite the significant stimulus to inflammation and the presence of connective tissue infiltration, the electroporation injury does not seem to compromise the subsequent muscle recovery in the *SJL/J* dysferlin-deficient mouse. Instead, a remarkable regenerative capacity of the skeletal muscle was observed when compared to WT mice. *SJL/J* exhibited higher levels of expression of regenerative genes at the earlier time point after injury (day 3) as compared to WT mice. Myogenic factors were much more upregulated, indicating a vigorous cellular response after injury. In addition, enhanced regeneration was confirmed through the intense expression of the developmental isoform of dMyHC, proving the formation of new myofibers. At later time points, mRNA expression followed a similar pattern but slightly elevated in normal muscle. Together, our findings suggest that dysferlin deficiency affects muscle recovery modifying the time course of the regenerative response. We could suggest that the faster regeneration in dysferlin-deficient muscle would be due to ongoing regenerative events in the intrinsic dystrophic process, already exposed to degeneration and regeneration cycles (Maley et al. 1994; Anderson et al. 1995). A preactive state in the dystrophic muscle could lead, at least in young dystrophic mice, to the prompt response observed, while WT muscle, not previously activated, requires to be stimulated to initiate the myogenic response. It would be a possible compensation for early muscle damage in young mice, but later it would change as time goes by and the chronic dystrophic process was installed (Weller et al. 1997). Furthermore, good muscle strength was observed in patients with dysferlinopathy before the onset of muscle weakness (Biondi et al. 2013). Also, a recent report (Hofhuis et al. 2017) described a greater exercise capacity in dysferlin-null mice compared to wild-type.

Our findings agree with previous studies that demonstrated a faster and more extensive myogenic response in *SJL/J* compared to *Balb/c*. Interestingly, at the beginning of the 1990s, when the *SJL/J* mouse was still not known as a dystrophic dysferlin-deficient model, some studies of regeneration has been done, using different methods of experimental injury. In all of them, good myogenesis and rapid regeneration in relation to other strains were observed (McGeachie and Grounds 1995). This efficient regeneration was not caused by differences in macrophages phenotype (Mitchell et al. 1995), but the host environment in muscle grafted experiments was determinant in the better regenerative process in these mice. Also, a better pattern of regeneration was observed in *SJL/J* mice when comparing the regenerative response with *BALBc* strains after the cut and crush muscle lesions (Grounds and McGeachie 1989).

Experiments using whole muscle grafts pointed to a faster myogenic development both in *SJL/J* autografts and in allografts from *BALBc* put into *SJL/J* mice, indicating that the regenerative response is host dependent, being *SJL/J* mice those with the best ability to regenerate (Roberts et al. 1997). Additional piece of evidence comes from the work by Mitchell et al. (1992, 1995), who showed that the muscle regenerative capacity might come from proper characteristics of the muscle itself, once *SJL/J* mice which had their bone marrow cells depleted and replaced by those from *BALBc* mice, displayed better muscle regeneration after crush-injury than the controls.

In contrast, other authors suggested that the lack of dysferlin results in slower myogenesis after severe contraction-induced injuries in dysferlin-deficient *A/J* mice (Roche et al. 2008). In another study, analysis of notexin-injured *C57BL/10-SJL.Dysf* muscles showed impaired regeneration, however, it was suggested that it is not at the level of myogenic factors expression, but it is associated with perturbed recruitment of neutrophils and failure to clear necrotic debris (Chiu et al. 2009).

It is characteristic, a normal resistance to physical activities before the onset of symptoms in dysferlinopathies. Despite this initial picture, the scenario tends to change with the progress of the disease (Biondi et al. 2013). The severity of symptoms appears more pronounced in humans when compared to animal models (Cohen et al. 2012) and it is also important to note that several factors can account to the discrepancies observed among the studies (Kobayashi et al. 2012). The dynamics of skeletal muscle regeneration produced by a different types of injury, age of the animals, methodologies and experimental controls are variable, making difficult a comparison among studies (Czerwinska et al. 2012). The use of different methodologies to induce degeneration can result in large experimental variation since alterations in muscle recovery mechanism depend on the type of muscle injury. In a study that involved two degrees of muscle injury, Roche et al. (2010) showed that the recovery from large-strains lengthening contractions involves necrosis followed by myogenesis activation in dysferlin-deficient muscles, whereas WT muscles perform membrane repair without significant myogenesis. On the other hand, the recovery from small strains is performed by myogenesis in both.

It is also noteworthy the importance of genetic background modulating the regenerative mechanism. The insertion of the dysferlin mutation in the background *C57BL/6* accentuates the disease, while in the *129/Sv* background, a milder phenotype was observed, highlighting the effect of modifying genes in the phenotypic manifestation (Demonbreun et al. 2016). Similarly, in a recent study in our laboratory, we transferred the *mdx* mutation to the *129/Sv* strain with the aim to create a more severe model for Duchenne Muscular Dystrophy, but surprisingly this new model showed amelioration of phenotype (Calyjur et al. 2016). Therefore, genetic modifiers can alter significantly the primary genetic mutation and should be considered to explain discrepancies among studies and the variable phenotype observed in humans. Further studies to examine different sources of strains and backgrounds under the same experimental conditions will be needed to address these questions.

The dysferlin-FAM65B-HDAC6 tricomplex

The identification of protein complexes has contributed progressively to better understand the pathological pathways of neuromuscular disorders. Dysferlin is known to interact with various proteins to perform its role in membrane repair (Matsuda et al. 2001; Lennon et al. 2003; Huang et al. 2005, 2007; Cai et al. 2009). However, proteins that interact with dysferlin related to its function in myogenesis are still little investigated. For this reason, the two recently identified cytoplasmatic proteins that interact with dysferlin at the beginning of myoblast differentiation, FAM65B, and HDAC6, deserve more attention. FAM65B interacts transiently with both HDAC6 and dysferlin and is essential for forming this tricomplex, important for the progression of myogenesis (Balasubramanian et al. 2014). HDAC6, a histone deacetylase, acts on deacetylation of acetylated α-tubulin present in polymerized form of microtubules. With the increase of dysferlin during differentiation, lower levels of "free" HDAC6 provide higher acetylation of α-tubulin and consequent elongation of myotubes (Di Fulvio et al. 2011). Inhibition of FAM65b or HDAC6 does not alter DYSF expression. During muscle regeneration in vivo, however, it is still unknown whether dysferlin deficiency can alter the expression of FAM65B and HDAC6. Thus, here we analyzed the mRNA expression of *Hdac6* and *Fam65b* at different time points of dysferlin-deficient and WT mice muscle recovery following electrical injury. Our results showed that these two genes are activated during regeneration considering mRNA and/or protein expressions. The peak of mRNA expression at day 3 coincided in *SJL/J* and WT muscle. In support of these findings, we observed high levels of protein expression at days 3 and 5 after injury in both strains. Similar to our results, *Fam65b* was previously reported peaking at 2–4 days after cardiotoxin-injured normal mouse muscle (Balasubramanian et al. 2014).

Interestingly, however, when comparing the expression level, we found a higher level in *SJL/J*. *Fam65b* was significantly higher at day 3 and 5 and *Hdac6* on day 5, showing that dysferlin deficiency affects FAM65B and HDAC6 expression during muscle regeneration. Higher expression of these two proteins could favor the formation of the tricomplex in the scenario of a small amount of dysferlin, allowing the process of regeneration in this model, as observed

in fact, by the success of the recovery of the SJL/J muscle after lesion.

In conclusion, our results suggest that dysferlin deficiency alters the time course of regeneration with the expression of myogenic factors peaking earlier after electroporation-induced muscle injury. The deficiency of dysferlin seems to impact the expression of the tricomplex components, suggesting a possible compensatory mechanism activated at the beginning of the regenerative process. In addition, this is the first study of the molecular regulation of *Hdac6* and *Fam65b* in models of muscle regeneration applied to dysferlinopathic mice and it points to the possible regulation of these components by dysferlin.

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Compliance with ethical standards

Conflict of interest The authors declare that they do not have any competing or financial interests.

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