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General Article

GENERAL ARTICLE

Sarcolipin deletion in *mdx* mice impairs calcineurin signalling and worsens dystrophic pathology

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Abstract

Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy affecting 1 in 3500 live male births. Although there is no cure for DMD, therapeutic strategies aimed at enhancing calcineurin signalling and promoting the slow fibre phenotype have shown promise in *mdx* mice, which is the classical mouse model for DMD. Sarcolipin (SLN) is a small protein that regulates the sarco(endo)plasmic reticulum Ca^{2+}-ATPase pump and its expression is highly upregulated in dystrophic skeletal muscle. We have recently shown that SLN in skeletal muscle amplifies calcineurin signalling thereby increasing myofibre size and the slow fibre phenotype. Therefore, in the present study we sought to determine the physiological impact of genetic *Sln* deletion in *mdx* mice, particularly on calcineurin signalling, fibre-type distribution and size and dystrophic pathology. We generated an *mdx*/*Sln*-null (*mdx*/Sln^{KO}) mouse colony and hypothesized that the soleus and diaphragm muscles from these mice would display blunted calcineurin signalling, smaller myofibre sizes, an increased proportion of fast fibres and worsened dystrophic pathology compared with *mdx* mice. Our results show that calcineurin signalling was impaired in *mdx*/Sln^{KO} mice as indicated by reductions in utrophin, stabilin-2 and calcineurin expression. In addition, *mdx*/Sln^{KO} muscles contained smaller myofibres, exhibited a slow-to-fast fibre-type switch that corresponded with reduced expression of mitochondrial proteins and displayed a worsened dystrophic pathology compared with *mdx* muscles. Altogether, our findings demonstrate a critical role for SLN upregulation in dystrophic muscles and suggest that SLN can be viewed as a potential therapeutic target.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle wasting disorder affecting 1 in 3500 live male births (1). Currently, there is no cure for DMD and most therapeutic strategies aim to improve quality of life while slowing disease trajectory. Calcineurin, a serine/threonine Ca^{2+}-dependent phosphatase, has emerged as a therapeutic target as studies using *mdx* mice, the classical DMD mouse model (2), demonstrate that enhancing calcineurin signalling alleviates the dystrophic phenotype (3–10). Specifically, calcineurin positively impacts muscular dystrophy in a multifactorial manner including an increase in the slow twitch type I fibres that are less prone to the dystrophic phenotype (11,12), an increase in utrophin expression that enhances sarcolemmal membrane stability (3,4,9,13–16) and an increase in the muscles' regenerative capacity (8,17–21). Furthermore, calcineurin may also alleviate the dystrophic phenotype by increasing myofibre size (22), however, there are some discrepant results regarding calcineurin's role in regulating muscle mass (23–28).

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We have recently shown that sarcolipin (SLN), a regulator of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump (29–32), amplifies calcineurin signalling in muscle thereby increasing the number of type I fibres, augmenting average myofibre cross-sectional area (CSA) and ultimately improving muscle function and structure in atrophic/myopathic conditions (33,34). In addition, we have recently shown that SLN's activation of calcineurin is critical for the adaptive responses that occur within a functionally overloaded plantaris (34). In the absence of SLN, functionally overloaded plantaris muscles fail to hypertrophy and transition towards the slow-oxidative phenotype as a result of impaired calcineurin signalling (34). Thus, our recent work has uncovered an important role for SLN in regulating adaptive changes in fibre-type composition and size through its effects on calcineurin signalling, which could prove beneficial for muscular dystrophy.

Interestingly, we and others have found SLN protein to be highly upregulated in skeletal muscles from *mdx* mice (Supplementary Material, Fig. S1 and 35); however, the physiological importance of this increase in SLN expression in the *mdx* model remains unknown. In the present study, we sought to determine the physiological role of SLN in *mdx* skeletal muscles by generating an *mdx*/*Sln*-null (*mdx*/Sln^{KO}) mouse line. Based on our previous findings demonstrating the importance of SLN in amplifying calcineurin signalling and muscle remodelling (33,34), we hypothesized that genetic deletion of *Sln* in *mdx* mice would result in blunted calcineurin signalling, reduced type I fibre counts, smaller myofibres and worsened dystrophic pathology.

Results

SLN deletion enhances SERCA's affinity for Ca^{2+} in soleus and diaphragm muscles

Western blotting for SLN protein in *mdx* soleus and diaphragm muscles shows that *Sln* was successfully deleted from *mdx* mice (Fig. 1A). Assessments of SERCA activity in soleus and diaphragm homogenates show that while *Sln* deletion did not improve maximal SERCA activity in either muscle (Fig. 1B), it did improve SERCA's apparent affinity for Ca^{2+} as indicated by a leftward shift in the activity curves and a significant increase in the Ca^{2+} concentration required to elicit 1/2 Vmax (presented as the negative logarithm of Ca^{2+}, pCa_{50}) pCa_{50} in both muscles (Fig. 1C–E). Finally, western blot analyses did not reveal any significant differences in SERCA1a or SERCA2a protein expression between the *mdx* and *mdx*/Sln^{KO} mice (Fig. 1F and G).

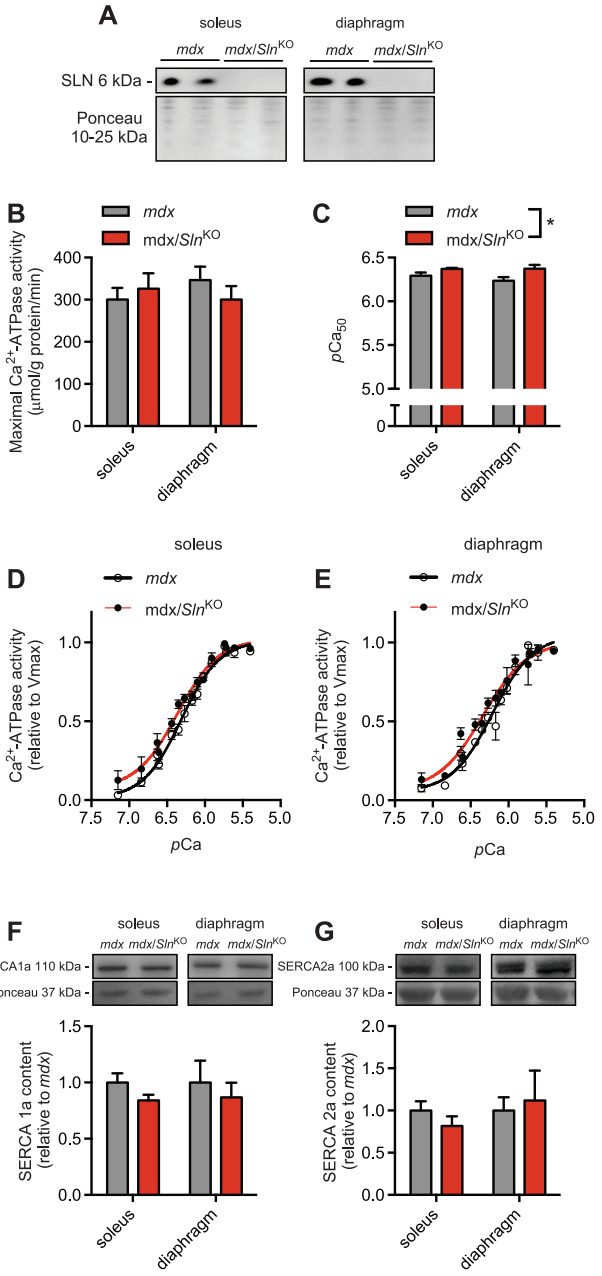

Figure 1. *Sln* deletion as depicted by western blotting **(A)** does not alter maximal SERCA activity **(B)** but does enhance SERCA's apparent affinity for Ca^{2+} in soleus and diaphragm muscles **(C–E)** from *mdx* mice. Western blot analyses of SERCA1a **(F)** and SERCA2a **(G)** did not reveal any significant differences. For (C), *denotes a significant main effect when using a two-way ANOVA, $P \leq 0.05$, n = 5–6 per group.

Calcineurin signalling is blunted in muscles from *mdx*/Sln^{KO} mice

Utrophin and stabilin-2 protein expressions are largely controlled by calcineurin signalling in muscle (13,21), and therefore, we used them as indicators of calcineurin activation. Our western blot analyses show that both utrophin and stabilin-2 expression were significantly lowered in soleus and diaphragm muscles from *mdx*/Sln^{KO} mice compared with *mdx* (Fig. 2A and B). Furthermore, we also found a significant reduction in calcineurin expression in muscles from *mdx*/Sln^{KO} mice compared with *mdx* (Fig. 2C).

SLN deletion alters fibre-type composition and size in *mdx* mice

We next examined the effects of *Sln* deletion on fibre-type composition and CSA within the *mdx* mouse model using immunofluorescent microscopy (Fig. 3A and B). With respect to fibre size, we found that myofibre CSA was significantly reduced in the *mdx*/Sln^{KO} soleus and diaphragm across all fibre types (Fig. 3C and D). With respect to fibre-type composition, both the soleus and diaphragm muscles underwent a slow-to-fast fibre-type shift in the *mdx*/Sln^{KO} mice compared with *mdx*. Specifically, we saw a significant reduction in type I fibre counts in the *mdx*/Sln^{KO} soleus (Fig. 3E), whereas, in the diaphragm, we observed a significant reduction in type IIA fibres and a significant increase in type IIX in the *mdx*/Sln^{KO} mice compared with *mdx* (Fig. 3F). Corresponding with the slow-to-fast fibre-type shift, we also found a significant reduction in the mitochondrial

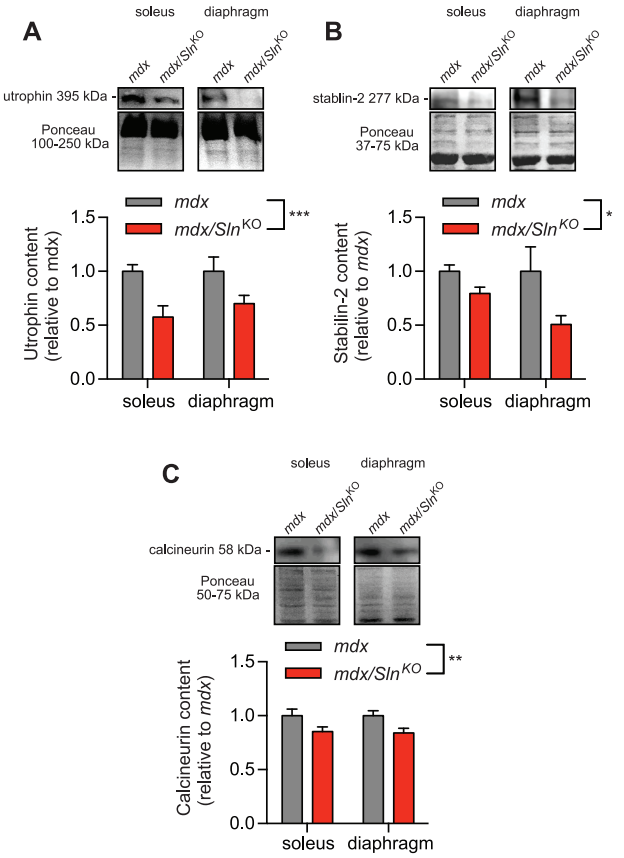

Figure 2. Calcineurin signalling in *mdx*/*Sln*^{KO} muscles is impaired as revealed with lowered utrophin, **(A)** stabilin-2 **(B)** and calcineurin **(C)** protein expression (n = 4–6 per group). *Denotes a significant main effect when using a two-way ANOVA, $P \leq 0.05$.

protein cytochrome c oxidase subunit IV (COX IV) (Fig. 3G) and a trending reduction in cytochrome c (Fig. 3H) in *mdx*/*Sln*^{KO} mice compared with *mdx*.

Worsened dystrophic pathology in muscles from *mdx*/*Sln*^{KO} mice

Finally, we tested whether dystrophic pathology would be worse in *mdx*/*Sln*^{KO} muscles compared with *mdx*. In this respect, serum creatine kinase (CK) was significantly elevated in *mdx*/*Sln*^{KO} mice compared with *mdx* (Fig. 4A). In addition, haematoxylin and eosin (H&E) staining revealed greater variability in fibre size with a significant increase in the coefficient of variation of the minimal Feret's diameter, which is a strong indicator of dystrophic pathology (36) (Fig. 4B and C). We also found a significant reduction in central nuclei counts (Fig. 4D) and a trending increase in muscle degeneration across the soleus and diaphragm from *mdx*/*Sln*^{KO} mice compared with *mdx* (Fig. 4E). After examining cage activity patterns with comprehensive laboratory animal monitoring system (CLAMS; Oxymax series; Columbus Instruments, Columbus, OH, USA), we found that *mdx*/*Sln*^{KO} mice were significantly less active compared with *mdx* mice (Fig. 4F).

Discussion

In the present study, we examined the physiological significance of SLN in the *mdx* mouse model where SLN protein was previously found to be highly upregulated (Supplementary Material, Fig. S1 and 35). Based on our recent findings (33,34), we hypothesized that SLN would play a critical role in activating calcineurin and launching compensatory cellular signalling events that combat the dystrophic pathology in the *mdx* mouse. After generating the *mdx*/*Sln*^{KO} mouse line we discovered that *Sln* deletion enhanced SERCA function, while impairing calcineurin signalling, leading to a slow-to-fast fibre-type shift, smaller myofibre CSA, worsened dystrophic pathology and reduced total cage activity.

Reductions in utrophin, stabilin-2 and calcineurin expression are altogether indicative of lowered calcineurin signalling in the *mdx*/*Sln*^{KO} muscles compared with *mdx*. Along with calcineurin expression, we chose utrophin and stabilin-2 as our markers of calcineurin signalling in muscle since the expression of these proteins in muscle are largely controlled by calcineurin (13,21). More importantly, these proteins are critical in combating dystrophic pathology, and therefore serve as functional measures of calcineurin signalling that could explain why dystrophic pathology was worsened in the *mdx*/*Sln*^{KO} mice. Utrophin is a dystrophin homolog that is upregulated in the *mdx* mouse to provide compensatory membrane stability, and mice lacking both dystrophin and utrophin display far worse dystrophic pathology when compared with *mdx* mice (14,16). Thus, the lowered utrophin expression not only indicates blunted calcineurin signalling, but also likely contributes to the augmented myofibre breakdown revealed with elevated serum CK levels and muscle degeneration in the *mdx*/*Sln*^{KO} mice. Stabilin-2 is a phosphatidylserine receptor that is important for myoblast fusion, and genetic deletion of stabilin-2 leads to smaller myofibre CSA and impaired muscle regeneration (21). Therefore, our findings of lowered stabilin-2 expression are not only indicative of blunted calcineurin signalling, but could also explain the reductions in myofibre CSA and central nuclei counts, which mark muscle regeneration. Indeed, calcineurin signalling is critical for muscle regeneration (8,17–21) and we have recently uncovered a role for SLN in mediating muscle regeneration after acute cardiotoxin injury (37).

The slow-to-fast fibre-type shift observed in the *mdx*/*Sln*^{KO} muscles could also be viewed as another indicator of impaired calcineurin signalling since calcineurin plays a critical role in activating the slow-oxidative phenotype (13,28,38–44). Correspondingly, we also found reduced COX IV and cytochrome c protein in the *mdx*/*Sln*^{KO} muscles, which is consistent with calcineurin's effect of enhancing mitochondrial content and respiratory function (45,46). It has been shown that promoting the oxidative phenotype can ameliorate muscular dystrophy (47) and it is well known that fast glycolytic fibres are more prone to dystrophic pathology compared with the slow-oxidative fibres (11,12). While glycolytic fibres generate more force thereby rendering them more susceptible to muscle damage in dystrophic conditions (12), it is also important to note that calcineurin signalling is much lower in these fibres compared with the slow-oxidative fibres (48). Indeed, utrophin expression has been highly linked to the slow-oxidative phenotype (13). Thus, our results also support the notion that faster fibres are more vulnerable to DMD or lack of dystrophin, as the slow-to-fast fibre-type shift observed in the *mdx*/*Sln*^{KO} muscles was associated with worsened dystrophic pathology and reduced cage activity.

Since muscular dystrophy is characterized by extensive degeneration and regeneration cycles, an increase in fibre size variability serves as an accurate indicator of dystrophic pathology. Here, we show that *mdx*/*Sln*^{KO} muscles have greater variation in the minimal Feret's diameter, which is a robust

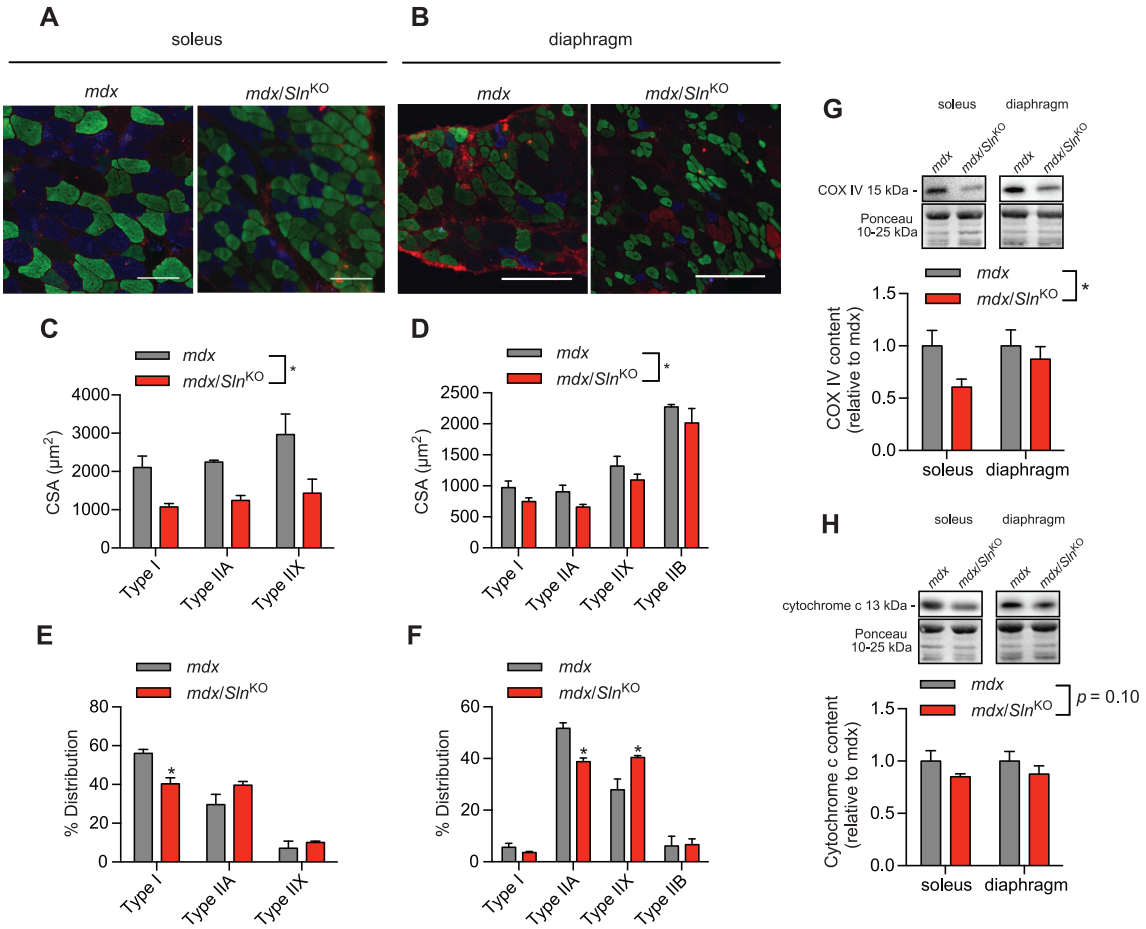

Figure 3. Immunofluorescent fibre typing **(A and B)** revealed a significant reduction in myofibre CSA **(C and D)** and a slow-to-fast fibre-type shift **(E and F)** in response to *Sln* deletion in the *mdx* mice (n = 3–4 per group). Western blotting demonstrated a significant reduction in COX IV **(G)** and a trending reduction in cytochrome c **(H)** in muscles from *mdx*/*Sln*^{KO} mice compared with *mdx*. For (C), (D), (G) and (H), *denotes a significant main effect when using a two-way ANOVA, $P \leq 0.05$.for (E) and (F), *denotes significance using a Student's *t*-test comparing *mdx* versus *mdx*/*Sln*^{KO} muscles, $P \leq 0.05$.

measure of myofibre size. In addition to this, we also found a trending increase in muscle degeneration with the percent of areas occupied by mononuclear cell and connective tissue infiltration (6). Furthermore, we and others have found that *mdx* mice are ~25% less active in their cages compared with wild-type (WT) mice likely as a result of overt muscle weakness (Supplementary Material, Fig. S2 and 49). Thus, our findings of reduced cage activity in the *mdx*/*Sln*^{KO} mice compared with *mdx* mice further support the worsened dystrophic myopathy in these animals, and this may not be explained by a less active phenotype observed solely in the *Sln*^{KO} mice since we have previously shown similar cage activity patterns between WT and *Sln*^{KO} mice (50).

While there is some considerable debate regarding calcineurin's role in regulating myofibre size (23–28), calcineurin's stimulation of myoblast fusion (18–21) can increase myofibre CSA by increasing the relative amount of nuclei per fibre (20,51). We have recently published findings in other mouse models that support SLN's role in regulating adaptive changes in myofibre size through calcineurin signalling (33,34). In another mouse model of muscle myopathy, we found that SLN was highly upregulated in the soleus muscles, and genetic deletion of *Sln* worsened the myopathic phenotype leading to smaller myofibres, as a result of impaired calcineurin signalling and lowered stabilin-2 (33). We also observed similar findings in soleus muscles subjected to tenotomy, which represents a model of soleus unloading (34). Conversely, in response to overloading stimuli, we recently found that plantaris muscles from *Sln*^{KO} mice failed to hypertrophy as a result of impaired calcineurin signalling and lowered stabilin-2 expression (34). Altogether, our findings of smaller myofibre CSA in the *mdx*/*Sln*^{KO} muscles compared with *mdx* are consistent with our previously published reports and further demonstrate the importance of SLN in maintaining muscle size across several myopathic/atrophic conditions.

Mechanistically, our study demonstrates that SLN activates calcineurin signalling and combats dystrophic pathology in *mdx* muscles by inhibiting SERCA function. Specifically, in the absence of SLN, soleus and diaphragm muscles from *mdx*/*Sln*^{KO} mice displayed an increase in SERCA's apparent affinity for Ca^{2+}. Presumably, higher SERCA activity at sub-maximal Ca^{2+} concentrations would lead to lower cytosolic Ca^{2+} levels and less activation of calmodulin and thus calcineurin. The improvement in SERCA's apparent affinity for Ca^{2+} is consistent with SLN's well-known role as a SERCA modulator but was not associated with any changes in SERCA isoform expression. Although, inconsistent with the slow-to-fast fibre-type switch observed in the *mdx*/*Sln*^{KO} muscles (52), this finding is in agreement with the notion that SERCA-myosin heavy chain (MHC) mismatch may occur under several myopathic conditions (53–55). Interestingly,

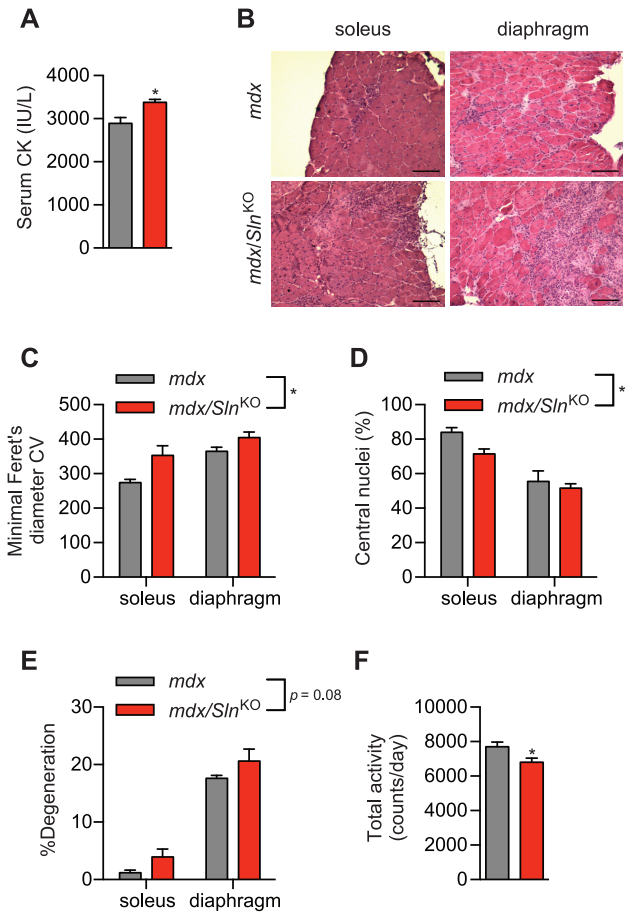

Figure 4. The *mdx*/Sln^{KO} muscles display worsened dystrophic pathology compared with *mdx* muscles. **(A)** Serum CK levels from *mdx*/Sln^{KO} and *mdx* mice. **(B)** H&E staining showing worsened dystrophic myopathy in *mdx*/Sln^{KO} muscles with increased variability in the minimal Feret's diameter, **(C)** lowered central nuclei counts **(D)** and increased muscle degeneration (% mononuclear cell and connective tissue infiltration; **E**). Total cage activity using CLAMS revealed a significant reduction in total activity in *mdx*/Sln^{KO} mice compared with *mdx*. For (A) and (F), *denotes significance using a Student's *t*-test comparing *mdx* versus *mdx*/Sln^{KO} muscles, $P \leq 0.05$; for (C-E), *denotes a significant main effect when using a two-way ANOVA, $P \leq 0.05$. For CK and histological analyses, n = 3–4 per group; and for total activity, n = 8–13.

previous studies have shown that improving SERCA function via SERCA overexpression can mitigate muscular dystrophy by reducing cytosolic Ca^{2+} overload and the ensuing muscle damage (56–58). We speculate that differences in the level of SERCA improvement could explain these discrepant results, as *Sln* deletion may not improve SERCA function to the extent of SERCA overexpression. Indeed, we only observed an improvement in SERCA's apparent affinity for Ca^{2+} with no significant alterations in maximal activity, whereas SERCA1 overexpression was shown to enhance maximal SERCA activity 2–3-fold in *mdx* muscles (57). Thus, our findings suggest that the subtle improvement in SERCA function observed with *Sln* deletion may not overcome the Ca^{2+} overload in *mdx* muscles, but rather results in an exacerbated dystrophic phenotype by reducing calcineurin signalling. Similar findings were observed after overexpressing parvalbumin in the *mdx* soleus, where increasing the content of this calcium buffering protein worsened the dystrophic pathology by impairing calcineurin activation (10).

While our study was under review, another study was published demonstrating that genetic deletion of *Sln* alleviated the dystrophic pathology and enhanced muscle function in the *mdx/utrophin* double knockout mouse (59). We believe that these discrepant findings can be explained by the fact that muscles obtained from *mdx/utrophin* double knockout mice display greater SLN upregulation when compared with *mdx* mice (35). Given that our findings show a critical role for SLN in mediating utrophin expression via calcineurin signalling in the *mdx* mouse, the greater SLN expression in the *mdx/utrophin* double knockout mouse likely represents a compensatory event attempting to increase utrophin protein. However, in the absence of the *utrophin* gene, the greater upregulation of SLN likely becomes a maladaptive response that further impairs SERCA function and Ca^{2+} handling and thus contributing to the worsened dystrophic pathology in the *mdx/utrophin* double knockout mouse. Thus, our results show that when *utrophin* expression remains intact, as with *mdx* mice and DMD patients, SLN upregulation is an adaptive response to increase utrophin expression and combat muscular dystrophy.

To date, there is no cure for DMD, and therapeutic strategies are aimed towards improving quality of life while slowing disease trajectory, and the results presented here reveal SLN as a potential therapeutic target. In this respect, treatment of *mdx* mice with corticosteroids, which is one of the therapies currently being used in the management of DMD, has been shown to alleviate dystrophic myofibre pathology via calcineurin activation and increased utrophin expression (60). Since corticosteroids also increase *Sln* expression (61), it stands to reason that the activation of calcineurin signalling in corticosteroid-treated skeletal muscle may be dependent on upregulation of SLN expression. Similarly, high-fat feeding has been shown to improve muscle function in *mdx* mice (62), and we and others have shown that high-fat feeding increases SLN expression by 3–5-fold (63,64). Thus, to further demonstrate SLN's therapeutic potential in the treatment of muscular dystrophy, we are currently investigating whether the benefits provided to *mdx* mice from corticosteroid treatment and high-fat feeding are mediated by SLN and its downstream effects on calcineurin signalling.

In our study we questioned whether the *mdx* soleus and diaphragm muscles would be affected differently with *Sln* deletion, and although we did not detect any significant interactions with our two-way analyses of variance (ANOVAs) we found that the average reduction in myofibre CSA was significantly greater in the *mdx*/Sln^{KO} soleus compared with diaphragm (−48 ± 6% versus −17 ± 3%, $P = 0.0004$). Similarly, we found that the increase in fibre size variability (soleus, 1.28 ± 0.10-fold versus diaphragm, 1.11 ± 0.04-fold, $P = 0.17$) and the reduction in central nuclei (soleus, −15 ± 3% versus diaphragm, −7 ± 4%, $P = 0.21$) was greater in the soleus compared with diaphragm; however, neither were statistically significant. Taken together, these data suggest that *Sln* deletion may impact the soleus muscle more than the diaphragm. Although purely speculative, this could be due to the fact that the disease phenotype is more severe in the *mdx* diaphragm relative to the soleus.

One limitation to our study is that we only assessed muscle pathology in mice at 3–6 months of age. This age was selected in order to bypass the initial necrosis and regeneration stage observed within the first 4 weeks of the *mdx* mouse (65,66). Thus, to fully comprehend the physiological role of SLN in the *mdx* mouse, future studies should examine the effects of *Sln* deletion in the *mdx* mouse across all ages, particularly in older *mdx* mice (20 months old) where the phenotype more closely resembles DMD pathology (67). Nevertheless, our present results show the importance of SLN protein in combatting dystrophic pathology in young adult *mdx* mice.

In summary, our study demonstrates for the first time that upregulation of SLN in the *mdx* mouse represents an adaptive response set out to enhance calcineurin signalling. Altogether, our findings suggest that SLN could be a novel therapeutic target in the management of muscular dystrophy, while also adding further evidence in support of calcineurin's therapeutic potential for muscle disease. With SLN's critical role in combatting muscle pathology in *mdx* mice, we propose that further increasing SLN protein may alleviate the dystrophic pathology in *mdx* mice and perhaps in DMD patients, and this will be examined further with future studies.

Materials and Methods

Animals

Sln ^{KO} mice were described previously (68) and were a kind gift from Dr Muthu Periasamy. To generate the *mdx*/*Sln*^{KO} and *mdx* colonies, homozygous *mdx* females ($X^{mdx}X^{mdx}$) were purchased from the Jackson Laboratory (001801, Bar Harbor, ME, USA) and crossed with homozygous *Sln*^{KO} males. The resulting F1 progeny were hemizygous *mdx* males ($X^{mdx}Y$) and heterozygous mdx females ($X^{mdx}X$) that were all heterozygous for *Sln* deletion. Experimental *mdx* and *mdx*/*Sln*^{KO} (50:50, C57BL6/J:C57BL10) mice were obtained after crossing the hemizygous *mdx* males with heterozygous *mdx* females from F1. All animals used in the study were adult mice ranging from 3 to 6 months of age. Animals were housed in an environmentally controlled room with a standard 12:12 hour light–dark cycle and allowed access to food and water *ad libitum*. All animal procedures were reviewed and approved by the Animal Care Committee of the University of Waterloo and are consistent with the guidelines established by the Canadian Council on Animal Care.

Tissue collection

All mice were sacrificed by cervical dislocation and the diaphragm and soleus muscles were isolated and either homogenized in homogenizing buffer (250 mM sucrose, 5 mM HEPES, 0.2 mM PMSF, 0.2% [w/v] NaN_3) or embedded in optimal cutting temperature (O.C.T.) compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) frozen in liquid nitrogen-cooled isopentane and then stored at −80°C until further analysis.

SERCA activity assay

SERCA activity was measured in muscle homogenates over Ca^{2+} concentrations ranging from *p*Ca 7.2 to 5.4 in the presence of the Ca^{2+} ionophore A23187 (C7522, Sigma Aldrich, St. Louis, MO, USA) using a Ca^{2+}-dependent, enzyme-linked spectrophotometric plate reader assay that has been described previously (69). Maximal SERCA activity was taken from the raw data, whereas SERCA's apparent affinity for Ca^{2+} was measured with the pCa_{50}. The pCa_{50} is the negative logarithm of the [Ca^{2+}] required to elicit half-maximal SERCA activity and was obtained from SERCA activity-*p*Ca curves. The SERCA activity-*p*Ca curves were generated with GraphPad Prism™ (Graphpad Software, La Jolla, CA, USA) by non-linear regression curve fitting, using an equation for a general cooperative model for substrate activation.

Western blotting

Western blotting was performed to determine expression levels of SLN, SERCA1a, SERCA2a, utrophin, calcineurin, stabilin-2, COX IV and cytochrome c in soleus and diaphragm muscles. The primary antibody directed against SLN was generated by Lampire Biological Laboratories (Everett, PA, USA) (70). The SERCA1a antibody (A52) was a kind gift from Dr David MacLennan (University of Toronto), whereas the SERCA2a antibody (MA3-919) was purchased from ThermoFisher Scientific (Waltham, MA, USA). The utrophin (610896) antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA), and the primary antibodies for calcineurin and stabilin-2 were obtained from Milliopore (Burlington, MA, USA) (07-1491) and Biorbyt (orb158499), respectively. Both primary antibodies for COX IV (sc-69630) and cytochrome c (sc-13156) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Quantitation of optical densities was performed using GeneTools (Syngene, Frederick, MD, USA) and values were normalized to total protein after ponceau staining.

Histochemical and immunofluorescent staining

Soleus and diaphragm muscles embedded in O.C.T. compound were cut into 10 µm thick cryosections with a cryostat (Thermo Fisher Scientific) maintained at −20°C. H&E staining was performed and images were acquired with a brightfield Nikon (Melville, NY, USA) microscope linked to a PixeLink digital camera (Navitar, Rochester, NY, USA). ImageJ software was used to examine central nuclei, muscle degeneration (percent of area occupied by mononuclear and connective tissue infiltration (6)) and the minimal Feret's diameter, which is the minimum distance of parallel tangents at opposing borders of the muscle fibre (36).

Immunofluorescence analysis of MHC expression was performed as previously described (71) with primary antibodies against MHCI, MHCIIa and MHCIIb to assess fibre-type distribution. Slides were visualized with an Axio Observer Z1 fluorescent microscope equipped with standard red, green, blue filters, an AxioCam HRm camera and AxioVision software (Carl Zeiss, Jena, Germany). Quantification of fibre distribution and CSA was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Serum CK analysis

Mice were anaesthetized using somnotol (0.65 mg/kg body weight) and blood from the left ventricle was drawn into a syringe. Blood was centrifuged at 5000*g* for 8 min and the serum was decanted and stored at −80°C until analysis. CK activity was measured using a kinetic fluorometric assay as previously described (72).

Cage activity

Daily cage activity was measured using CLAMS as previously described (50). This system is equipped with X- and Z-axes infrared photocell detectors that allow monitoring total cage activity. Mice were placed in the CLAMS for a 3 day period for three separate trials and had free access to food and water.

Statistics

All values are presented as standard error of the mean. Comparisons between *mdx* and *mdx*/*Sln*^{KO} mice were performed using either a Student's *t*-test or two-way ANOVA. Since, dystrophic pathology differs across soleus and diaphragm muscles from *mdx* mice with the diaphragm presenting with a more severe phenotype, we also examined the potential interaction effect

between genotype and muscle type with a two-way ANOVA. Statistical significance was set to $P \leq 0.05$.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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References

1. Emery, A.E. (2002) The muscular dystrophies. *Lancet*, **359**, 687–695.
2. Bulfield, G., Siller, W.G., Wight, P.A. and Moore, K.J. (1984) X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. USA*, **81**, 1189–1192.
3. Angus, L.M., Chakkalakal, J.V., Mejat, A., Eibl, J.K., Belanger, G., Megeney, L.A., Chin, E.R., Schaeffer, L., Michel, R.N. and Jasmin, B.J. (2005) Calcineurin-NFAT signaling, together with GABP and peroxisome PGC-1{alpha}, drives utrophin gene expression at the neuromuscular junction. *Am. J. Physiol. Cell Physiol.*, **289**, C908–C917.
4. Chakkalakal, J.V., Harrison, M.A., Carbonetto, S., Chin, E., Michel, R.N. and Jasmin, B.J. (2004) Stimulation of calcineurin signaling attenuates the dystrophic pathology in mdx mice. *Hum. Mol. Genet.*, **13**, 379–388.
5. Sakuma, K. and Yamaguchi, A. (2010) The functional role of calcineurin in hypertrophy, regeneration, and disorders of skeletal muscle. *J. Biomed. Biotechnol.*, **2010**, 721219.
6. Stupka, N., Gregorevic, P., Plant, D.R. and Lynch, G.S. (2004) The calcineurin signal transduction pathway is essential for successful muscle regeneration in mdx dystrophic mice. *Acta Neuropathol.*, **107**, 299–310.
7. Stupka, N., Michell, B.J., Kemp, B.E. and Lynch, G.S. (2006) Differential calcineurin signalling activity and regeneration efficacy in diaphragm and limb muscles of dystrophic mdx mice. *Neuromuscul. Disord.*, **16**, 337–346.
8. Stupka, N., Schertzer, J.D., Bassel-Duby, R., Olson, E.N. and Lynch, G.S. (2008) Stimulation of calcineurin Aalpha activity attenuates muscle pathophysiology in mdx dystrophic mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **294**, R983–R992.
9. Chakkalakal, J.V., Michel, S.A., Chin, E.R., Michel, R.N. and Jasmin, B.J. (2006) Targeted inhibition of Ca2+ /calmodulin signaling exacerbates the dystrophic phenotype in mdx mouse muscle. *Hum. Mol. Genet.*, **15**, 1423–1435.
10. Zein, M.A., Chin, E.R., Jasmin, B.J. and Michel, R.N. (2012) Targeted expression of the non-native Ca+2-buffering protein parvalbumin exacerbates the dystrophic phenotype in mdx mouse slow muscle fibers. *FASEB J.*, **26**, 1086.1023. Published abstract..
11. Webster, C., Silberstein, L., Hays, A.P. and Blau, H.M. (1988) Fast muscle fibers are preferentially affected in Duchenne muscular dystrophy. *Cell*, **52**, 503–513.
12. Consolino, C.M. and Brooks, S.V. (2004) Susceptibility to sarcomere injury induced by single stretches of maximally activated muscles of mdx mice. *J. Appl. Physiol. (1985)*, **96**, 633–638.
13. Chakkalakal, J.V., Stocksley, M.A., Harrison, M.A., Angus, L.M., Deschenes-Furry, J., St-Pierre, S., Megeney, L.A., Chin, E.R., Michel, R.N. and Jasmin, B.J. (2003) Expression of utrophin A mRNA correlates with the oxidative capacity of skeletal muscle fiber types and is regulated by calcineurin/NFAT signaling. *Proc. Natl. Acad. Sci. USA*, **100**, 7791–7796.
14. Deconinck, A.E., Rafael, J.A., Skinner, J.A., Brown, S.C., Potter, A.C., Metzinger, L., Watt, D.J., Dickson, J.G., Tinsley, J.M. and Davies, K.E. (1997) Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. *Cell*, **90**, 717–727.
15. Grady, R.M., Teng, H., Nichol, M.C., Cunningham, J.C., Wilkinson, R.S. and Sanes, J.R. (1997) Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell*, **90**, 729–738.
16. Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M. and Davies, K. (1998) Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat. Med.*, **4**, 1441–1444.
17. Chen, H.H., Chen, W.P., Yan, W.L., Huang, Y.C., Chang, S.W., Fu, W.M., Su, M.J., Yu, I.S., Tsai, T.C., Yan, Y.T. *et al.* (2015) NRIP is newly identified as a Z-disc protein, activating calmodulin signaling for skeletal muscle contraction and regeneration. *J. Cell Sci.*, **128**, 4196–4209.
18. Horsley, V., Friday, B.B., Matteson, S., Kegley, K.M., Gephart, J. and Pavlath, G.K. (2001) Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. *J. Cell Biol.*, **153**, 329–338.
19. Horsley, V., Jansen, K.M., Mills, S.T. and Pavlath, G.K. (2003) IL-4 acts as a myoblast recruitment factor during mammalian muscle growth. *Cell*, **113**, 483–494.
20. Horsley, V. and Pavlath, G.K. (2003) Prostaglandin F2(alpha) stimulates growth of skeletal muscle cells via an NFATC2-dependent pathway. *J. Cell Biol.*, **161**, 111–118.
21. Park, S.Y., Yun, Y., Lim, J.S., Kim, M.J., Kim, S.Y., Kim, J.E. and Kim, I.S. (2016) Stabilin-2 modulates the efficiency of myoblast fusion during myogenic differentiation and muscle regeneration. *Nat. Commun.*, **7**, 10871.
22. Hudson, M.B. and Price, S.R. (2013) Calcineurin: a poorly understood regulator of muscle mass. *Int. J. Biochem. Cell Biol.*, **45**, 2173–2178.
23. Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. *et al.* (2001) Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.*, **3**, 1014–1019.
24. Serrano, A.L., Murgia, M., Pallafacchina, G., Calabria, E., Coniglio, P., Lomo, T. and Schiaffino, S. (2001) Calcineurin controls nerve activity-dependent specification of slow skeletal muscle fibers but not muscle growth. *Proc. Natl. Acad. Sci. USA.*, **98**, 13108–13113.
25. Parsons, S.A., Millay, D.P., Wilkins, B.J., Bueno, O.F., Tsika, G.L., Neilson, J.R., Liberatore, C.M., Yutzey, K.E., Crabtree, G.R., Tsika, R.W. *et al.* (2004) Genetic loss of calcineurin blocks mechanical overload-induced skeletal muscle fiber type switching but not hypertrophy. *J. Biol. Chem.*, **279**, 26192–26200.

26. Parsons, S.A., Wilkins, B.J., Bueno, O.F. and Molkentin, J.D. (2003) Altered skeletal muscle phenotypes in calcineurin Aalpha and Abeta gene-targeted mice. *Mol. Cell. Biol.*, **23**, 4331–4343.
27. Dunn, S.E., Chin, E.R. and Michel, R.N. (2000) Matching of calcineurin activity to upstream effectors is critical for skeletal muscle fiber growth. *J. Cell Biol.*, **151**, 663–672.
28. Naya, F.J., Mercer, B., Shelton, J., Richardson, J.A., Williams, R.S. and Olson, E.N. (2000) Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J. Biol. Chem.*, **275**, 4545–4548.
29. Asahi, M., Kurzydlowski, K., Tada, M. and MacLennan, D.H. (2002) Sarcolipin inhibits polymerization of phospholamban to induce superinhibition of sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs). *J. Biol. Chem.*, **277**, 26725–26728.
30. Odermatt, A., Becker, S., Khanna, V.K., Kurzydlowski, K., Leisner, E., Pette, D. and MacLennan, D.H. (1998) Sarcolipin regulates the activity of SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca2+-ATPase. *J. Biol. Chem.*, **273**, 12360–12369.
31. Gamu, D., Bombardier, E., Smith, I.C., Fajardo, V.A. and Tupling, A.R. (2014) Sarcolipin provides a novel muscle-based mechanism for adaptive thermogenesis. *Exerc. Sport Sci. Rev.*, **42**, 136–142.
32. Gorski, P.A., Glaves, J.P., Vangheluwe, P. and Young, H.S. (2013) Sarco(endo)plasmic reticulum calcium ATPase (SERCA) inhibition by sarcolipin is encoded in its luminal tail. *J. Biol. Chem.*, **288**, 8456–8467.
33. Fajardo, V.A., Gamu, D., Mitchell, A., Bloemberg, D., Bombardier, E., Chambers, P.J., Bellissimo, C., Quadrilatero, J. and Tupling, A.R. (2017) Sarcolipin deletion exacerbates soleus muscle atrophy and weakness in phospholamban overexpressing mice. *PLoS One*, **12**, e0173708.
34. Fajardo, V.A., Rietze, B.A., Chambers, P.J., Bellissimo, C., Bombardier, E., Quadrilatero, J. and Tupling, A.R. (2017) Effects of sarcolipin deletion on skeletal muscle adaptive responses to functional overload and unload. *Am. J. Physiol. Cell Physiol*, **313**, C154–C161.
35. Schneider, J.S., Shanmugam, M., Gonzalez, J.P., Lopez, H., Gordan, R., Fraidenraich, D. and Babu, G.J. (2013) Increased sarcolipin expression and decreased sarco(endo)plasmic reticulum Ca2+ uptake in skeletal muscles of mouse models of Duchenne muscular dystrophy. *J. Muscle Res. Cell Motil.*, **34**, 349–356.
36. Briguet, A., Courdier-Fruh, I., Foster, M., Meier, T. and Magyar, J.P. (2004) Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscul. Disord.*, **14**, 675–682.
37. Bellissimo, C., Bombardier, E., Chambers, P., Fajardo, V.A. and Tupling, A.R. (2016) Sarcolipin ablation impairs muscle regeneration after acute injury. *FASEB J.*, **30**, 1224.1228. Published abstract.
38. Berchtold, M.W., Brinkmeier, H. and Muntener, M. (2000) Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. *Physiol. Rev.*, **80**, 1215–1265.
39. Bigard, X., Sanchez, H., Zoll, J., Mateo, P., Rousseau, V., Veksler, V. and Ventura-Clapier, R. (2000) Calcineurin co-regulates contractile and metabolic components of slow muscle phenotype. *J. Biol. Chem.*, **275**, 19653–19660.
40. Chin, E.R., Olson, E.N., Richardson, J.A., Yang, Q., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R. and Williams, R.S. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev.*, **12**, 2499–2509.
41. Long, Y.C., Glund, S., Garcia-Roves, P.M. and Zierath, J.R. (2007) Calcineurin regulates skeletal muscle metabolism via coordinated changes in gene expression. *J. Biol. Chem.*, **282**, 1607–1614.
42. McCullagh, K.J., Calabria, E., Pallafacchina, G., Ciciliot, S., Serrano, A.L., Argentini, C., Kalhovde, J.M., Lomo, T. and Schiaffino, S. (2004) NFAT is a nerve activity sensor in skeletal muscle and controls activity-dependent myosin switching. *Proc. Natl. Acad. Sci. USA*, **101**, 10590–10595.
43. Schiaffino, S. and Serrano, A. (2002) Calcineurin signaling and neural control of skeletal muscle fiber type and size. *Trends Pharmacol. Sci.*, **23**, 569–575.
44. Swoap, S.J., Hunter, R.B., Stevenson, E.J., Felton, H.M., Kansagra, N.V., Lang, J.M., Esser, K.A. and Kandarian, S.C. (2000) The calcineurin-NFAT pathway and muscle fiber-type gene expression. *Am. J. Physiol. Cell Physiol.*, **279**, C915–C924.
45. Ryder, J.W., Bassel-Duby, R., Olson, E.N. and Zierath, J.R. (2003) Skeletal muscle reprogramming by activation of calcineurin improves insulin action on metabolic pathways. *J. Biol. Chem.*, **278**, 44298–44304.
46. Jiang, L.Q., Garcia-Roves, P.M., de Castro Barbosa, T. and Zierath, J.R. (2010) Constitutively active calcineurin in skeletal muscle increases endurance performance and mitochondrial respiratory capacity. *Am. J. Physiol. Endocrinol. Metab.*, **298**, E8–E16.
47. Selsby, J.T., Morine, K.J., Pendrak, K., Barton, E.R. and Sweeney, H.L. (2012) Rescue of dystrophic skeletal muscle by PGC-1alpha involves a fast to slow fiber type shift in the mdx mouse. *PLoS One*, **7**, e30063.
48. Schiaffino, S. and Reggiani, C. (2011) Fiber types in mammalian skeletal muscles. *Physiol. Rev.*, **91**, 1447–1531.
49. Tatem, K.S., Quinn, J.L., Phadke, A., Yu, Q., Gordish-Dressman, H. and Nagaraju, K. (2014) Behavioral and locomotor measurements using an open field activity monitoring system for skeletal muscle diseases. *J. Vis. Exp.*, **91**, 51785.
50. Bombardier, E., Smith, I.C., Gamu, D., Fajardo, V.A., Vigna, C., Sayer, R.A., Gupta, S.C., Bal, N.C., Periasamy, M. and Tupling, A.R. (2013) Sarcolipin trumps beta-adrenergic receptor signaling as the favored mechanism for muscle-based diet-induced thermogenesis. *FASEB J.*, **27**, 3871–3878.
51. Allen, D.L., Roy, R.R. and Edgerton, V.R. (1999) Myonuclear domains in muscle adaptation and disease. *Muscle Nerve*, **22**, 1350–1360.
52. Tupling, A.R. (2004) The sarcoplasmic reticulum in muscle fatigue and disease: role of the sarco(endo)plasmic reticulum Ca2+-ATPase. *Can. J. Appl. Physiol.*, **29**, 308–329.
53. Hamalainen, N. and Pette, D. (2001) Myosin and SERCA isoform expression in denervated slow-twitch muscle of euthyroid and hyperthyroid rabbits. *J. Muscle Res. Cell Motil.*, **22**, 453–457.
54. Salanova, M., Schiffl, G. and Blottner, D. (2009) Atypical fast SERCA1a protein expression in slow myofibers and differential S-nitrosylation prevented by exercise during long term bed rest. *Histochem. Cell Biol.*, **132**, 383–394.
55. Talmadge, R.J., Roy, R.R., Bodine-Fowler, S.C., Pierotti, D.J. and Edgerton, V.R. (1995) Adaptations in myosin heavy chain profile in chronically unloaded muscles. *Basic Appl Myol*, **5**, 117–137.
56. Goonasekera, S.A., Lam, C.K., Millay, D.P., Sargent, M.A., Hajjar, R.J., Kranias, E.G. and Molkentin, J.D. (2011) Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. *J. Clin. Invest.*, **121**, 1044–1052.

57. Mazala, D.A., Pratt, S.J., Chen, D., Molkentin, J.D., Lovering, R.M. and Chin, E.R. (2015) SERCA1 overexpression minimizes skeletal muscle damage in dystrophic mouse models. *Am. J. Physiol. Cell Physiol.*, **308**, C699–C709.
58. Morine, K.J., Sleeper, M.M., Barton, E.R. and Sweeney, H.L. (2010) Overexpression of SERCA1a in the mdx diaphragm reduces susceptibility to contraction-induced damage. *Hum. Gene Ther.*, **21**, 1735–1739.
59. Voit, A., Patel, V., Pachon, R., Shah, V., Bakhutma, M., Kohlbrenner, E., McArdle, J.J., Dell'Italia, L.J., Mendell, J.R., Xie, L.H. *et al.* (2017) Reducing sarcolipin expression mitigates Duchenne muscular dystrophy and associated cardiomyopathy in mice. *Nat. Commun.*, **8**, 1068.
60. St-Pierre, S.J., Chakkalakal, J.V., Kolodziejczyk, S.M., Knudson, J.C., Jasmin, B.J. and Megeney, L.A. (2004) Glucocorticoid treatment alleviates dystrophic myofiber pathology by activation of the calcineurin/NF-AT pathway. *FASEB J.*, **18**, 1937–1939.
61. Gayan-Ramirez, G., Vanzeir, L., Wuytack, F. and Decramer, M. (2000) Corticosteroids decrease mRNA levels of SERCA pumps, whereas they increase sarcolipin mRNA in the rat diaphragm. *J. Physiol.*, **524 (**Pt 2**)**, 387–397.
62. Radley-Crabb, H.G., Fiorotto, M.L. and Grounds, M.D. (2011) The different impact of a high fat diet on dystrophic mdx and control C57Bl/10 mice. *PLoS currents*, **3**, RRN1276.
63. Bal, N.C., Maurya, S.K., Sopariwala, D.H., Sahoo, S.K., Gupta, S.C., Shaikh, S.A., Pant, M., Rowland, L.A., Bombardier, E., Goonasekera, S.A. *et al.* (2012) Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat. Med.*, **18**, 1575–1579.
64. Bombardier, E., Smith, I.C., Vigna, C., Fajardo, V.A. and Tupling, A.R. (2013) Ablation of sarcolipin decreases the energy requirements for Ca2+ transport by sarco(endo)plasmic reticulum Ca2+-ATPases in resting skeletal muscle. *FEBS Lett.*, **587**, 1687–1692.
65. Carnwath, J.W. and Shotton, D.M. (1987) Muscular dystrophy in the mdx mouse: histopathology of the soleus and extensor digitorum longus muscles. *J. Neurol. Sci.*, **80**, 39–54.
66. Dangain, J. and Vrbova, G. (1984) Muscle development in mdx mutant mice. *Muscle Nerve*, **7**, 700–704.
67. Lefaucheur, J.P., Pastoret, C. and Sebille, A. (1995) Phenotype of dystrophinopathy in old mdx mice. *Anat. Rec.*, **242**, 70–76.
68. Babu, G.J., Bhupathy, P., Timofeyev, V., Petrashevskaya, N.N., Reiser, P.J., Chiamvimonvat, N. and Periasamy, M. (2007) Ablation of sarcolipin enhances sarcoplasmic reticulum calcium transport and atrial contractility. *Proc. Natl. Acad. Sci. USA*, **104**, 17867–17872.
69. Duhamel, T.A., Green, H.J., Stewart, R.D., Foley, K.P., Smith, I.C. and Ouyang, J. (2007) Muscle metabolic, SR Ca(2+) -cycling responses to prolonged cycling, with and without glucose supplementation. *J. Appl. Physiol. (1985)*, **103**, 1986–1998.
70. Fajardo, V.A., Bombardier, E., Vigna, C., Devji, T., Bloemberg, D., Gamu, D., Gramolini, A.O., Quadrilatero, J. and Tupling, A.R. (2013) Co-expression of SERCA isoforms, phospholamban and sarcolipin in human skeletal muscle fibers. *PLoS One*, **8**, e84304.
71. Bloemberg, D. and Quadrilatero, J. (2012) Rapid determination of myosin heavy chain expression in rat, mouse, and human skeletal muscle using multicolor immunofluorescence analysis. *PLoS One*, **7**, e35273.
72. Szasz, G., Gruber, W. and Bernt, E. (1976) Creatine kinase in serum: 1. Determination of optimum reaction conditions. *Clin. Chem*, **22**, 650–656.