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Abstract

The sarcoplasmic reticulum (SR) plays an important role in calcium homeostasis. SR calcium mishandling is described in pathological conditions such as myopathies. Here, we investigated whether the nuclear receptor subfamily 1 group D member (NR1D1, also called REV-ERBα) regulates skeletal muscle SR calcium homeostasis. Our data demonstrate that NR1D1 deficiency in mice impairs SERCA-dependent SR calcium uptake. NR1D1 acts on calcium homeostasis by repressing the SERCA inhibitor myoregulin through direct binding to its promoter. Restoration of myoregulin counteracts the effects of NR1D1 overexpression on SR calcium content. Interestingly, myoblasts from Duchenne myopathy patients display lower NR1D1 expression, whereas pharmacological NR1D1 activation ameliorates SR calcium homeostasis, and improves muscle structure and function in dystrophic mdx/Utr+/− mice. Our findings demonstrate that NR1D1 regulates muscle SR calcium homeostasis, pointing to its therapeutic interest for mitigating myopathy.

Keywords: NR1D1 / calcium / endoplasmic reticulum / myoregulin / skeletal muscle / REV-ERBα
Introduction

Skeletal muscle is not only required for movements, but is also crucial for other vital functions such as respiration. Myopathies (1,2), among which Duchenne muscular dystrophy (DMD) is one of the most prevalent forms, result in progressive muscle weakness and wasting, and can lead to premature death. Despite progress in gene therapy, DMD still remains an unmet medical need, calling for new strategies to alleviate skeletal muscle degeneration.

Calcium (Ca\(^{2+}\)) is important for muscle contractile function and its subcellular distribution is tightly regulated by several pumps and channels (3). Ca\(^{2+}\) is stored in the endoplasmic/sarcoplasmic reticulum (ER/SR) where it mainly interacts with Ca\(^{2+}\)-binding proteins such as calsequestrin (4). Following an action potential, membrane depolarization triggers a massive Ca\(^{2+}\) release through the Ryanodine Receptor (RyR), hence promoting contraction and muscle force generation. Ca\(^{2+}\) reuptake from the cytosol into the SR lumen by the Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA) allows muscle relaxation and a new cycle of contraction/relaxation. Because SERCA Ca\(^{2+}\) pump activity plays a prominent role in skeletal muscle contractility, it is tightly regulated by different factors including the recently discovered inhibitory SR transmembrane alpha helix micropeptide myoregulin (MLN) (5). Disturbances of these fine-tuned processes have been observed in DMD, where chronically elevated cytosolic Ca\(^{2+}\) concentrations (6,7), decreased SERCA activity (8–10) and reduced Ca\(^{2+}\) release upon excitation can be observed (2,9,11). Progressive loss of muscle force generation, as observed in the mdx mouse model of DMD, is explained by the absence of homeostatic return to basal cytosolic Ca\(^{2+}\) levels between two contractions (12), underlying the importance of normal SERCA activity for muscle function.

We have previously reported that the druggable nuclear receptor subfamily 1 group D member NR1D1, a transcriptional repressor also known as REV-ERB\(\alpha\) (13), improves skeletal muscle
function and exercise capacity (14). Especially, NR1D1 improves mitochondrial function along with increased mitochondrial biogenesis (14). We investigated here whether NR1D1 controls additional mechanisms accounting for skeletal homeostasis. We particularly assessed whether NR1D1 modulates the major SR function, i.e., Ca^{2+} handling. We demonstrate that NR1D1, through its transcriptional repressive activity on the Mln gene, increases SERCA activity and SR Ca^{2+} content in mouse and human muscle cells. Importantly, pharmacological NR1D1 activation with SR9009 decreases Mln expression, improves calcium handling, enhances force generation and minimizes tissue damage in severely dystrophic mdx/utr^{+/-} mice. Overall, our results identify NR1D1 as a new regulator of SR calcium homeostasis that may represent a therapeutic target in skeletal muscle disorders related to impaired reticular calcium homeostasis, such as myopathies.

Results

NR1D1 improves muscle force along with SR Ca^{2+} homeostasis

We first aimed to determine whether NR1D1 is important for muscle force generation and found that muscle contraction is reduced by ~50% in Nr1d1^-/- mice compared to their wild-type (Nr1d1^+/+) littermate controls (Figure 1A, Supplemental Figure 1A). Since Ca^{2+} homeostasis is essential for muscle force generation, we next determined whether NR1D1 controls SR Ca^{2+} handling. Muscle microsomes, i.e., sarcoplasmic vesicles, were prepared from Nr1d1^-/- and Nr1d1^+/+ littermates. SERCA-dependent SR Ca^{2+} uptake capacity was measured over time after the addition of Ca^{2+} pulses or thapsigargin (TG), a potent inhibitor of SERCA activity (15), by using a fluorescent probe detecting extramicrosomal Ca^{2+}. The slope of fluorescence decrease, i.e., SR Ca^{2+} uptake, was significantly lower in Nr1d1^-/- mice compared to Nr1d1^+/+ mice, revealing a reduction in SERCA activity in absence of NR1D1 (Figures 1B and 1C). By contrast, in vivo treatment of wild-type mice with the NR1D1 agonist SR9009 led to improved SERCA activity
measured in muscle microsomes prepared from these mice (Figures 1D and 1E). In order to measure passive Ca\textsuperscript{2+} release from SR as a surrogate of its initial Ca\textsuperscript{2+} content, differentiated NR1D1-overexpressing or control (pBabe) C2C12 myotubes were loaded with the cytosolic Ca\textsuperscript{2+}-sensitive probe Fluo4-AM and then challenged with TG to release Ca\textsuperscript{2+} from the SR. TG addition led to a greater elevation in Fluo4 fluorescence in NR1D1-overexpressing cells compared to pBabe, revealing that NR1D1 overexpression is associated with increased SR Ca\textsuperscript{2+} content (Figures 1F and 1G). Consistently, similar results were obtained using the cytosolic Ca\textsuperscript{2+}-sensitive probe Fura-2 AM, which is a dual-excitation, single-emission Ca\textsuperscript{2+} indicator avoiding possible loading artifacts (Figures 1H and 1I). In line, basal cytosolic calcium, buffered at least in part by the SR, is reduced by NR1D1 overexpression (Figure 1J). In contrast, siRNA-mediated Nr1d1 silencing (Supplemental Figure 1B) decreases reticular calcium content in C2C12 myotubes (Figures 1K and 1L). Together, these data indicate that NR1D1 controls SR Ca\textsuperscript{2+} homeostasis in skeletal muscle.

**NR1D1 controls Ca\textsuperscript{2+} homeostasis through direct repression of myoregulin expression**

We then aimed to identify the mechanism by which NR1D1 regulates calcium homeostasis. Because skeletal muscle SR Ca\textsuperscript{2+} homeostasis is mainly controlled by the Ryanodine Receptor RyR1, SERCA1 and SERCA2, we determined whether NR1D1 controls their expression. Whereas Ryr1, Serca1 and Serca2 mRNA expression was identical in Nr1d1\textsuperscript{+/+} and Nr1d1\textsuperscript{-/-} mice (Figures 2A-2C) as well as in pBabe and NR1D1-overexpressing cells (Figures 2D-2F), expression of Mln, a recently identified skeletal muscle-specific SERCA inhibitor (5), was significantly higher in skeletal muscle from Nr1d1\textsuperscript{-/-} mice compared to control littermates (Figure 2G). In line, treatment with SR9009 in vivo to activate NR1D1 significantly decreased Mln expression (Figure 2H). Consistently, NR1D1 overexpression or NR1D1 pharmacological activation with SR9009
decreased $Mln$ expression in C2C12 cells (Figures 2I-2J), whereas $NR1D1$ targeting siRNA or cell treatment with the NR1D1 antagonist SR8278 induced $Mln$ expression (Figure 2K-2L).

*In silico* analysis identified at least three putative Rev-erb Response Elements (RevRE) located at 1.4, 5.4 and 6.7kb upstream the $Mln$ transcription start site (Figure 2M). Using Chromatin ImmunoPrecipitation (ChIP)-qPCR experiments performed on mouse skeletal muscle, we demonstrate that NR1D1 binds to these three regions (Figure 2N). To test whether direct NR1D1 binding to the $Mln$ gene is required for its regulation, we used skeletal muscle-specific mutant mice expressing a DNA binding domain (DBD)-deficient NR1D1 protein. As observed in the $Nr1d1^{-/-}$ mice, $Mln$ expression was higher in skeletal muscle-specific mutant mice compared to wild-type floxed littermates (Figure 2O). Altogether, these data reveal that NR1D1 represses $Mln$ gene expression by direct binding to its promoter.

To functionally demonstrate that MLN is key in the regulation of muscle Ca$^{2+}$ homeostasis by NR1D1, we aimed to restore MLN expression in NR1D1-overexpressing cells. As expected, overexpression of $MLN$ alone, by viral vector transduction in C2C12 myotubes, reduced SR Ca$^{2+}$ stores compared to control pBabe cells (Figures 2P and 2Q). More importantly, $MLN$ overexpression in $NR1D1$-overexpressing cells normalized Ca$^{2+}$ handling (Figures 2R and 2S).

**Pharmacological NR1D1 activation improves SERCA-dependent Ca$^{2+}$ uptake and alleviates the dystrophic phenotype in Duchenne Myopathy**

Calcium homeostasis is known to be impaired in several myopathies (2,16). To determine whether this could be due, at least in part, to a deregulation of NR1D1 and its downstream targets, we first analyzed its expression in publicly available microarray datasets from dystrophic muscles. Interestingly, we found that $NR1D1$ mRNA is expressed, albeit to significantly lower levels, in Duchenne Muscular Dystrophy (DMD) patients from different studies (17–19) (Figure 3A,
Supplemental Figures S2A-S2B). The same was observed in muscle biopsies from DMD patients (Supplemental Figure 2C). In line, NR1D1 expression was reduced by ~30% in DMD muscle cells compared to human control cells (Figure 3B). These data indicate altered NR1D1 expression, hence action, may be associated to the Duchenne dystrophy, pointing to NR1D1 as an interesting target specifically in this myopathy. Therefore, we tested whether pharmacological NR1D1 activation by SR9009 may improve Ca\(^{2+}\) homeostasis in primary cells from patients suffering from DMD. A significantly higher SR Ca\(^{2+}\) release triggered by TG was measured in SR9009-treated compared to vehicle-treated DMD cells (Figures 3C-D).

To determine whether this was related to MLN, we used immortalized DMD myotubes since they shared reduced NR1D1 expression and SR calcium content as observed in primary cells (Supplemental Figures 2D-2H). Whereas MLN over-expression in healthy myotubes reduces SR calcium content (Supplemental Figures 2I and 2J), shRNA against MLN improves SR calcium content in DMD immortalized myotubes (Supplemental Figures 2K and 2L).

To further assess whether pharmacological NR1D1 activation could improve muscle function in a pathological DMD model in vivo, mdx/Utr\(^{+/-}\) mice, which closely recapitulate the features of the human disease, were daily injected with SR9009 for 20 days. Histological analysis revealed that tissue architecture was improved in SR9009-treated mice (Figure 3E and Supplemental Figure 3A), along with a mild, but significant, decrease in small fibers and an increase in medium/large fibers compared to vehicle-injected mdx/Utr\(^{+/-}\) mice (Figure 3F). Circulating blood creatine phospho kinase (CPK), which is a marker of muscle damage, was strongly decreased in the SR9009-treated group compared to vehicle-treated mdx/Utr\(^{+/-}\) mice (Figure 3G). Several fibrosis markers, including Sirius red staining (Figure 3E), muscle hydroxyproline quantity (Figure 3H), Col1a2 (Figure 3I) and Pdgfra (Figure 3J) expression, were also reduced by SR9009 treatment. Next, we evaluated whether SR9009 treatment was able to improve muscle calcium homeostasis
and function in this model of myopathy. As expected, based on the data from the genetic models of deletion or over-expression of NR1D1 specifically in skeletal muscle, daily injection of SR9009 for 20 days reduced Mln expression in muscle from mdx/Utr+/− mice (Figure 3K), whereas SERCA activity was improved (Figure 3L), both being strongly correlated (Supplemental Figure 3B). More importantly, gastrocnemius muscle-developed contraction force was significantly ameliorated by SR9009 treatment in mdx/Utr+/− mice compared to vehicle-injected littermates (Figure 3M - Supplemental Figure 3C).

In conclusion, a 20-day NR1D1 agonist treatment improved SR homeostasis and muscle function in a mouse model of Duchenne myopathy.

Discussion

Our data demonstrate that NR1D1 improves calcium homeostasis in skeletal muscle by directly controlling Mln expression, hence SERCA activity. We also report that NR1D1 pharmacological activation by synthetic ligands may reveal therapeutic interests since it improves calcium homeostasis in human cells from DMD patients and alleviates the myopathy phenotype in mdx/Utr+/− mice.

RyR1 and SERCA1 are the two major SR proteins controlling Ca\(^{2+}\) fluxes in skeletal muscle. Nevertheless, neither RyR1 nor SERCA1 expression was impacted by NR1D1. Therefore, we focused on MLN, the main glycolytic/mixed muscle endogenous SERCA inhibitor (20). MLN is a recently discovered 46-amino acid micropeptide that forms a single transmembrane alpha helix and interacts with the skeletal muscle SERCA1 isoform to inhibit its pumping activity, thereby decreasing SR Ca\(^{2+}\) content (5). Here, we identified NR1D1 as a new direct transcriptional repressor of Mln gene expression. Indeed, we demonstrated that NR1D1 binds to three RevRE located in the Mln promoter via a functional DNA-binding domain. These data are potentially limited: since the anti-NR1D1 antibody used in our ChIP assay was ChIP grade, not ChIPseq
grade, non-specific binding to RevRE in the Mln promoter remains possible. By modulating Ca\(^{2+}\) handling, MLN was proposed to modulate skeletal muscle contractile activity and to represent a promising drug target for improving Ca\(^{2+}\)-related skeletal muscle disorders and muscle performance (5). Consistently, Mln deletion in mice improves skeletal muscle performance (5). Yet, modulators of Mln expression remained to be identified. Interestingly, pharmacological NR1D1 activation, which we have shown in previous studies to improve muscle performance in non-pathological contexts (14) and to block glucocorticoid-induced muscle wasting (21), is able to repress Mln expression. We were also able to strongly correlate Mln expression and calcium uptake activity, despite the absence of validation at the protein level due to the lack of specific anti-MLN commercially available antibodies. Therefore, we bring novel insights into the molecular mechanisms by which NR1D1 exerts beneficial effects on muscle function and uncover a novel pathway to control Mln, hence skeletal muscle Ca\(^{2+}\) handling and likely contractile function.

We have demonstrated that NR1D1 is expressed in DMD cells, albeit to a lower extent compared to control human myotubes, suggesting that increasing NR1D1 activity could represent a new therapeutic option in myopathies. In muscle of patients suffering from DMD, the absence of dystrophin causes muscular contraction impairment with altered Ca\(^{2+}\) handling, i.e., raised cytosolic Ca\(^{2+}\) concentrations and depletion of SR Ca\(^{2+}\) stores due to impaired uptake capacity (2,22). Here, we confirm these data and we further demonstrate that pharmacological activation of NR1D1 by a synthetic ligand improves SR Ca\(^{2+}\) content in human DMD myotubes. This was also observed in vivo in a DMD mouse model in which pharmacological activation of NR1D1 significantly improved muscle histology and reduced damage markers and fibrosis. By itself, and consistent with other studies showing that improving Ca\(^{2+}\) homeostasis mitigates DMD (7,22), we can hypothesize that pharmacological NR1D1 activation reduces MLN expression, which may
partly contribute to the improved muscle contractility observed in myopathic mice. Yet, we cannot rule out that NR1D1 activation exerts effects on endothelial cells, interstitial mesenchymal cells, immune cells or satellite cells, contributing to the overall improved phenotype.

Others reported that NR1D1 antagonism with SR8278 may also improve muscle function, reduce fibrosis and increase mitochondrial biogenesis in mdx mice (23). This study is in apparent contradiction with the present results and with results that, we and these authors, have previously published demonstrating that NR1D1 agonism with SR9009 improves muscle mitochondrial function and exercise capacity in healthy mice (14). In addition, we and others have reported that NR1D1 positively controls skeletal muscle mass by counteracting both autophagy (14) and proteasomal-associated fiber atrophy (21) and by promoting myoblast differentiation through mTORC1 signaling pathway activation (24), again supporting a positive action of NR1D1 in skeletal muscle. Although out of scope of this study, these cellular processes may also contribute to the improvement of the myopathy phenotype upon NR1D1 activation. Indeed, we also observed that Nr1d1−/− mice displayed impaired tetanic contraction, suggesting that calcium uptake impairment is not the only parameter explaining this muscular deficit. Compensatory mechanisms may interfere as both Nr1d2 overexpression and knock-down were reported to lead to a similar increase in mitochondrial biogenesis (25). Moreover, SR9009 as well as SR8278 target both NR1D1 and NR1D2 (26) and may also exert NR1D1-independent activities (27). In the present study, we have used genetic models of deletion or over-expression of NR1D1 specifically in skeletal muscle and in mouse and human myotubes to support our model and validate the role of NR1D1 in ameliorating muscle calcium handling and improving dystrophy. While this is possibly one reason for the apparent discrepancy between our results and others (23), it should also be noted that we used a different mouse model of muscle dystrophy. While the mdx mouse is widely used, it is a very mild model far from the human Duchenne myopathy phenotype (28). In contrast, we
used the \textit{mdx/Utr}^{+/-} model that presents a profound phenotype more relevant to the human situation, which may also explain why different results were obtained. We also showed, that this pertains to human muscle biopsies (17–19), although conclusions have to be taken cautiously due to patient age, stage and treatment heterogeneity, and in myotubes from Duchenne patients. Moreover, although it would be impossible to disentangle the two pathways, the beneficial effects of NR1D1 on muscle function could also be related to the combination of two mechanisms. Indeed, by improving mitochondrial function (14), NR1D1 activation could lead to higher ATP availability for calcium pumps and myofibrillar proteins. Even though, for the above-mentioned reasons, the current ligands cannot be used in the clinic, our results advocate for further development of more selective NR1D1-activating drugs, which could be of interest in the treatment of myopathies, and likely other muscle disorders characterized by altered Ca^{2+} homeostasis.

\textit{Methods}

\textbf{Study design}

In the primary objective of our study, genetically-engineered and pharmacologically-treated cells and mice were used to determine whether NR1D1 modulates calcium homeostasis in the SR. The translational impact of our finding was then tested in human muscle cells obtained from patients suffering from DMD and in a mouse model for Duchenne myopathy. Based on age and weight, animals were assigned to the different experimental groups. The number of samples for in vivo and in vitro assays was based on our experience and publications in the field.

\textbf{Cell culture and treatments}

\textit{Mouse and human muscle cells}

C2C12 cells (ATCC, Manassas, Virginia, USA) were cultured in high glucose DMEM (41965039, Gibco, Thermo Fischer Scientific, Waltham, Massachusetts, USA) supplemented with 10\% fetal
bovine serum and 0.4% gentamycin and differentiated by replacing the previous medium with DMEM supplemented with 2% horse serum and 0.4% gentamycin for 5 days. Primary myoblasts from control and DMD patients, kindly given by Myobank-AFM (Myology Institute, Pitié-Salpêtrière Hospital, Paris, France) were cultured in DMEM supplemented with 20% fetal calf serum and 0.2% primocin. Immortalized myoblasts from healthy and DMD patients, kindly given by MyoLine (Myology Institute, Pitié-Salpêtrière Hospital, Paris, France) were cultured in M199/DMEM (1V/3V) supplemented with 20% FBS, 25 µg/mL fetuin, 0.5 ng/mL bFGF, 5 ng/mL EGF, 5 µg/mL insulin, 0.2 µg/mL dexamethasone, and 50µg/mL gentamycin. Differentiation was initiated in DMEM containing 5 µg/mL insulin, 50µg/mL gentamycin.

**NR1D1 and Mln overexpression in C2C12**

Generation of NR1D1 and myoregulin (MLN)-overexpressing C2C12 cells was performed as previously described (5,14). Briefly, mouse Mln and human NR1D1 coding sequences were inserted into the pBabe plasmid (Addgene, Cambridge, Massachusetts, USA) by using BamHI-SalII restriction sites. NR1D1 and Mln or empty pBabe plasmids were transfected into Phoenix cells using JetPEI (Polyplus, Illkirch-Graffenstaden, France). Next, the supernatant of Phoenix cell culture was incubated with C2C12 cells, leading to their infection by retroviruses. The selection was done by a 15-day treatment with puromycin for NR1D1-overexpressing cells, and neomycin for Mln overexpressing cells.

**Lentivirus-mediated overexpression of MLN in human myoblast cell lines**

cDNA encoding human MLN was amplified by RT-PCR and subcloned into the pLenti-IRES-BSD vector via EcoRI and XhoI restriction sites to produce the recombinant lentivirus plasmid pLenti-hMRLN-IRES-BSD. The day before the transfection, 2.10^6 of 293T cells were plated in 10-cm culture dishes in DMEM proliferation medium supplemented with 10% FBS and 1% gentamycin. HEK-293T cells were transfected using the jetPEI Transfection Reagent with 9 µg of pLenti-IRES-
BSD or plenti-hMRLN-IRES-BSD vector. In both cases, cells were co-transfected with 4.5 μg of pMDL, 2.7 μg of VSVG, and 1.8 μg of pREV plasmids to permit the production of the viral particles. After 24 hours, media was replaced. 72 hours later supernatants containing viruses were collected and filtered through a 0.45-μm filter. Human immortalized myoblasts were plated the day before infection, and infected overnight at ~40% confluency with a media mixed 1:1 with viral supernatant (supplemented with Medium 199, Fetuin, bFGF, EGF, insulin, dexamethasone and FBS), in presence of 4 μg/mL polybrene (Sigma-Aldrich). 24 hours later, supernatant was removed and fresh medium was added. Infected myoblast cells were screened using 16 μg/ml blasticidin for 10 days. The remaining cells were evaluated for overexpression of human myoregulin by qPCR.

**Lentiviral knockdown of MLN in human DMD myoblast cell lines**

Stable MLN silencing in immortalized DMD human myoblasts was achieved using a Dhharmacon SMARTvector lentiviral shRNA delivery system according to manufacturer’s instructions. Briefly, cells were infected, in the presence of 4 μg/mL polybrene, with virus expressing a non-targeting control or MLN shRNA (MOI 50). Percentage of RFP positive cells was checked 48 hours after infection. SMARTvector Lentiviral Particles (catalog # V3SH7945-245505968 and V3SH7945-245224017) towards MLN targeted respectively ACCACTACCTGGGATTAAT and GGACTTCGCTTATTGAACC sequences. Non-Targeting SMARTvector shRNA Lentiviral particles (catalog # S-005000) were used as an infection control

**Pharmacological modulation of NR1D1 activity**

Pharmacological modulation of NR1D1 was obtained by adding in the culture medium either the synthetic agonist SR9009 (10μM) or the synthetic antagonist SR8278 (10μM), both from Merck
Chimie SAS, Fontenay-Sous-Bois, France. TG was dissolved in DMSO, which was added at the same concentration in control conditions.

**Mice housing and treatments**

All mice were housed in our animal facility with a 12h/12h light/dark cycle and had free access to food and water. *Nr1d1* -deficient mice (*Nr1d1*<sup>−/−</sup>) and skeletal muscle-specific NR1D1 DBD mutant mice (*Nr1d1*<sup>DBDmut<sub>fl/fl</sub>;MCK<sup>Cre/+</sup>) expressing a truncated NR1D1 lacking the DBD were generated as previously described (14,29) and compared to respective control littermates. 

*Gastrocnemius* muscles were collected at 2 pm and were either flash-frozen in liquid nitrogen or rapidly frozen using isopentane cooled with liquid nitrogen for immunostaining, or freshly processed for microsome preparation. The effect of pharmacological NR1D1 activation on SR calcium uptake was tested in gastrocnemius muscle collected from wild-type mice treated with SR9009 (100 mg/kg) or its vehicle twice daily for 3 days (21).

To evaluate the therapeutic potential of NR1D1 activation in Duchenne myopathy, twenty five-week old *mdx/Utr<sup>+/−</sup>* (30) mice were treated with SR9009 (100 mg/kg, once a day for 20 days) or vehicle. All the described procedures were approved by the local ethics committee (CEEA75).

**Creatine PhosphoKinase activity**

Blood CPK activity was measured with the creatine kinase assay kit (ab155901, Abcam, Cambridge, United Kingdom), according to manufacturer’s instructions.

**Muscular hydroxyproline assay**

4-hydroxyproline, a major component of collagen, was detected by the use the assay kit MAK008 (Sigma-Aldrich, St. Louis, Missouri, USA), according to manufacturer’s instructions. Briefly, muscle (10 mg) was homogenized in 100 µL of water and hydrolysis was started by adding 100 µL of 12 M HCl. After 3 hours at 120°C, samples were spun down at 10,000 g. 20 µL of the
resulting supernatant were transferred in a 96-well plate and evaporated under vacuum. 100 µL of chloramine T/oxidation buffer mixture was added into the wells. Then, 100 µL of DMAB reagent were added. Plate was incubated for 90 minutes at 60°C. Absorbance was measured at 560nm and compared to hydroxyproline standards.

**In Situ Contractile Properties of the Gastrocnemius Muscle**

Mice were deeply anesthetized with intraperitoneal injections of ketamine (50 mg.kg\(^{-1}\)) and dexmedetomidine (Domitor, 0.25 mg.kg\(^{-1}\)). The dissection protocol was previously described. Briefly, all the muscles of the right hindlimb were denervated, except the gastrocnemius muscle, which was isolated from surrounding tissues. Then, the limb was immersed in a bath of paraffin oil thermostatically controlled (37°C), and fixed with bars and pins. The gastrocnemius muscle was maintained in a horizontal position and its distal tendon was connected to a force transducer (Grass FT 10, Grass Instruments, West Warwick, Rhode Island, USA). The muscle length was adjusted to produce a maximal twitch peak tension (Pt). Contractions were induced by stimulation of the sciatic nerve (0.2ms pulses) through bipolar platinum electrodes at twice the minimum voltage required to obtain the maximal twitch response. At the end of the recording session, the muscle was removed for determination of muscle wet weight, frozen in liquid nitrogen and stored at −80°C.

**RT-qPCR analysis**

RNAs were extracted from mouse muscle, C2C12 and human myotubes seeded in 6-well plates or from gastrocnemius muscle, according to the Trizol (Invitrogen, Thermo Fischer Scientific, Waltham, Massachusetts, USA)/Chloroform/Isopropanol protocol. After DNase treatment, cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, California, USA). qPCRs were realized using SYBR® Green Real-Time PCR Master Mix kit (Agilent Technologies, Santa Clara, California, USA) and a MX3005 apparatus (Agilent
Technologies, Santa Clara, California, USA). Mouse and human-specific primers are recapitulated in supplemental tables S1 and S2, respectively. Gene expression was normalized to cyclophilin A (Ppia).

**SERCA-dependent Ca\(^{2+}\) uptake**

*Gastrocnemius* muscles were collected and homogenized at 4°C in a dedicated buffer (Tris-HCl pH7 1M, sucrose 8%, PMSF 1mM, DTT 2mM) with a Polytron (Kinematica AG, Malters Switzerland). Samples were then centrifuged at 1,300g at 4°C for 10min in order to remove nuclei. The supernatant obtained after a second centrifugation (20,000g, 4°C, 20min) corresponds to the enriched microsomal fraction. 150\(\mu\)g proteins were placed in calcium uptake buffer (CaCl\(_2\) 120\(\mu\)M, EGTA 150\(\mu\)M, Tris-HCl 30mM pH7, KCl 100mM, NaN\(_3\) 5mM, MgCl\(_2\) 6mM, oxalate 10mM) and put in 2mL-chambers of the Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) equipped with the fluorescence LED2-module. Calcium green probe (1\(\mu\)M) and ATP (5mM) were added and fluorescence was measured over time (\(\lambda_{\text{ex}}\) 506nm, \(\lambda_{\text{em}}\) 531nm). Ca\(^{2+}\) (10\(\mu\)M) pulse was then injected into the chambers. Finally, TG (1\(\mu\)M) was added in order to ensure that SERCA-dependent Ca\(^{2+}\) uptake was measured. We calculated the slope of the fluorescence intensity decrease subtracted with the residual slope measured in the presence of TG reflects SERCA-dependent Ca\(^{2+}\) uptake.

**SR Ca\(^{2+}\) content in C2C12 cells**

Experiments were conducted following a technical protocol adapted from Ducastel *et al.* (32). C2C12 cells and human myoblasts were plated in a 96-well plate (20,000/well) and differentiated for 4 days. Then, medium was replaced for 24 hours by low Ca\(^{2+}\) concentration Locke’s Buffer (NaCl 154mM, NaHCO\(_3\) 4mM, KCl 5mM, CaCl\(_2\) 2H\(_2\)O 0.1mM, MgCl\(_2\) 6H\(_2\)O 1mM, Glucose 5mM, Hepes 10mM, pH7.4), as previously described (33). To detect cytosolic Ca\(^{2+}\), myotubes were then loaded with Fluo4-AM (\(\lambda_{\text{ex}}\) 490nm, \(\lambda_{\text{em}}\) 516nm) for 30min at 37°C, with 5% CO\(_2\) in
free Ca\(^{2+}\) Locke’s buffer. Following 2 washes with 2.3mM Ca\(^{2+}\) Locke’s buffer, TG (1\(\mu\)M) was added in order to deplete SR Ca\(^{2+}\) store. Fluorescence intensity was immediately recorded every 10 seconds during 5min using a microplate reader (Infinite 200 pro, Tecan, Männedorf, Switzerland) in order to estimate SR Ca\(^{2+}\) content until stabilization.

**Calcium imaging**

Cells were grown on glass bottom dishes to carry out calcium imaging experiments. Ratiometric dye Fura-2/AM (F1221, Invitrogen, Thermo Fischer Scientific, Waltham, Massachusetts, USA) was used as a Ca\(^{2+}\) indicator. Cells were loaded with 2\(\mu\)M Fura-2/AM for 45 min at 37°C and 5% CO\(_2\) in corresponding medium and subsequently washed three times with external solution containing (in mM): 140 NaCl, 5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 5 Glucose, 10 Hepes (pH 7.4). The glass bottom dish was then transferred in a perfusion chamber on the stage of Nikon Eclipse Ti microscope (Nikon, Minato City, Tokyo, Japan). Fluorescence was alternatively excited at 340 and 380 nm with a monochromator (Polychrome IV, TILL Photonics GmbH, Kaufbeuren, Germany) and captured at 510 nm by a QImaging CCD camera (QImaging, Teledyne Photometrics, Tucson, Arizona, USA). Acquisition and analysis were performed with the MetaFluor 7.7.5.0 software (Molecular Devices Corp., San Jose, California, USA).

**Tissue histology**

Cross Sectional Area was analyzed as previously described (21,34). Conventional hematoxylin-eosin (HE) staining was performed to describe histological status of muscle sections (35). Sirius red staining was performed to describe fibrosis (36).

**ChIP experiment**

ChIP assays were performed as previously described (37) with minor modifications as follows. *Gastrocnemius* muscles from wild-type C57/Bl6 mice were homogenized in LB1 buffer (Hepes-
KOH 10mM pH7.5, NP-40 0.5%, MgCl₂ 5mM, DTT 500µM, Cytochalasin B 3µg/mL, protease inhibitor cocktail) and cross-linked with 1% paraformaldehyde for 10min at room temperature. Chromatin was sheared during 90min using the Bioruptor (Diagenode, Liège, Belgium) coupled to a watercooling system and subsequently concentrated with centricon 10kDa column (Millipore, Burlington, Massachusetts, USA). 50µg of chromatin were immunoprecipitated overnight at 4°C with an antibody against NR1D1 (13418S, ChIP grade, Cell signaling Technology, Danvers, Massachusetts, United States). BSA/yeast tRNA-blocked Protein A/G dynabeads (Invitrogen, Thermo Fischer Scientific, Waltham, Massachusetts, USA) were then added for 6h at 4°C while agitating and washed. Cross-linking was reversed by incubating precipitated chromatin overnight at 65°C. DNA was purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and was analyzed by qPCR using the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, California, USA) and specific primers (Supplemental Table S3).

Statistics

The number of biological replicates n, which was chosen on the basis of our experience and good laboratory practice, is reported in each figure legend. Values are means ± sem. The analysis was performed with GraphPad Prism software 9.0. One-way ANOVA followed by Tukey post-hoc tests are carried out in order to establish statistical significance when comparing three groups or more. Unpaired or paired Student t-tests were used to compare two groups, as indicated in figure legends. Significant effects are indicated as follows p<0.05 (*), p<0.01 (**), p<0.001 (***)

Data availability

The publicly available GSE data were analyzed by the GEO2R tool available on the NCBI website (https://www.ncbi.nlm.nih.gov/geo/geo2r/). Benjamini & Hochberg (False discovery rate) was applied to the p-values. Data were then analyzed on GraphPad Prism 9.0.
Study approval

All the described animal procedures were approved by the local ethics committee (CEEA75).

Author contributions

AB, CD and SL conceived and designed the experiments, AB, CD, AML, BP, YS, KK, AH, MG, CG, VM, SD, MC, QT, MZ, JB, AF and LF acquired and analyzed experiments, AB, CD, AML, BP, YS, KK, NP, FPR, BB, HD and SL interpreted data, AB, HD and SL wrote the original draft manuscript, AB, CD, BP, AML, YS, KK, NP, FPR, BB, BS, HD and SL reviewed and edited the manuscript, all authors approved the final version. AB and CD share the first co-authorship, AB is first in the order since he initiated the project and contributed to the original draft.

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*Conflict of interest*

The authors have declared that no conflict of interest exists.
References


Figure 1. NR1D1 regulates SR Ca\(^{2+}\) homeostasis in skeletal muscle.
(A) *In situ* measurement of gastrocnemius-developed force upon an electrical stimulus in wild-type (*Nr1d1+/+*) and *Nr1d1−/−* mice, ***p=0.0004 vs. *Nr1d1+/+, n=9-10, unpaired t-test. (B) Representative curves of SERCA-inhibitable Ca^{2+} uptake in microsomal fractions prepared from muscle from *Nr1d1+/+* and *Nr1d1−/−* mice. Decrease in fluorescence indicates Ca^{2+} uptake by microsomes. Arrows indicate Ca^{2+} or thapsigargin (TG) injections. Red rectangles were used for slope calculation of fluorescence decrease (C), indicative of specific SERCA Ca^{2+} uptake. Data are means ± SEM, n=6, *p=0.0203 vs. *Nr1d1+/+, unpaired t-test. (D) Representative curves of SERCA-inhibitable Ca^{2+} uptake in microsomes prepared from muscle from vehicle- or SR9009-treated wild-type mice. I Slopes of the decreasing fluorescence over time. Means ± SEM, n=4, *p=0.0408 vs. vehicle, unpaired t-test. (F) TG-induced SR Ca^{2+} release in control pBabe and *NR1D1*-overexpressing C2C12 myotubes. Cells were loaded with Fluo4-AM to detect cytosolic Ca^{2+}. SR Ca^{2+} content depletion was induced by TG (1µM). Means ± SEM of Delta F/F0 ratio, n=10. (G) Delta F/F0 ratio normalized to pBabe values, obtained 5min after TG-induced Ca^{2+} release and expressed as means ± SEM. n=10, **p=0.007 vs. pBabe, unpaired t-test. (H) Representative Fura-2/AM experiments (ratio F340/F380) in pBabe and *NR1D1*-overexpressing cells. (I) Normalized SR calcium concentration released upon TG treatment in pBabe and *NR1D1*-overexpressing cells. (J) Normalized basal cytosolic calcium concentration (mean of the 100 first seconds) in pBabe and *NR1D1*-overexpressing cells. Appearance in I and J is box and whiskers, showing all points with min and max (n>300 in each group). ***p<0.0001 vs. pBabe, unpaired t-test. (K) TG-induced SR Ca^{2+} release in C2C12 transfected by control (siCTRL) or *Nr1d1* siRNA (siNr1d1). Results are means ± SEM of Delta F/F0 ratio, n=17. (G) Delta F/F0 ratio normalized to siCTRL values, obtained 5min after TG-induced Ca^{2+} release. Means ± SEM. n=17, **p=0.0044 vs. siCTRL, unpaired t-test.
Figure 2. NR1D1 represses myoregulin (Mln) expression through direct binding to its promoter.
RyR1, Serca1 and Serca2 gene expression in muscle from Nr1d1+/+ and Nr1d1−/− mice (A-C, n=6), and in pBabe and NR1D1-overexpressing differentiated C2C12 (D-F, n=3). Mln expression in (G) muscle from Nr1d1+/+ and Nr1d1−/− mice (n=9, **p=0.0025 vs. Nr1d1+/+), (H) muscle from SR9009-treated wild-type (WT) animals (n=5, **p=0.0069 vs. vehicle), (I) NR1D1-overexpressing-C2C12 (n=5, **p=0.0023 vs. pBabe), (J) C2C12 treated with 10µM of the NR1D1 agonist SR9009 (n=3, *p=0.0155 vs. DMSO), (K) C2C12 transfected with siNr1dl (n=3, ***p<0.001 vs. siCTRL), (L) C2C12 treated with 10µM of the NR1D1 antagonist SR8278 (n=6, ***p<0.0001 vs. DMSO). A-L: means±SEM, unpaired t-test. (M) Schematic representation of the Mln promoter indicating three putative Rev-erba Response Elements (RevRE), located ~1.4, ~5.4kb and ~6.7kb upstream the transcription initiation site. (O) Chromatin ImmunoPrecipitation analysis using an anti-NR1D1 antibody or control Immunoglobulin G (IgG). n=6-8 mice, data are means ± SEM, site -6.7kb **p=0.0017, site -5.4kb ***p<0.0001, site -1.4kb ***p=0.001 vs. IgG. (N) Mln expression in mice with muscle-specific expression of a mutated isoform of NR1D1 lacking the DNA binding domain (Nr1d1 DBDmutβ/β, MCKCre/+ ) and control Nr1d1 DBDmutβ/β mice. n=3-5, *p=0.0139 vs. Nr1d1 DBDmutβ/β, unpaired t-test. (P) Thapsigargin (TG)-induced Sarcoplasmic Reticulum (SR) Ca2+ release in pBabe and Mln-overexpressing differentiated C2C12. Means ± SEM of Delta F/F0 ratio, n=7. (Q) Peak fluorescence intensity of TG-induced SR Ca2+ release in pBabe Mln-overexpressing differentiated C2C12, normalized to pBabe and expressed as mean ± SEM. n=7, **p=0.024 vs. pBabe, unpaired t-test. (R) TG-induced SR Ca2+ release in pBabe, NR1D1-overexpressing and NR1D1/Mln-overexpressing differentiated C2C12. Means ± SEM of Delta F/F0 ratio, n=6. (S) Peak fluorescence intensity of TG-induced SR Ca2+ release in pBabe, NR1D1-overexpressing and NR1D1/Mln-overexpressing differentiated C2C12, normalized to pBabe and displayed as means ± SEM. n=6, *p<0.026 vs. pBabe, $p<0.0293$ vs. NR1D1, 1-way ANOVA, Tukey’s multiple comparison test.
Figure 3. NR1D1 activation alleviates Duchenne muscular dystrophy features both in mice and human myoblasts.
(A) NR1D1 expression in muscle biopsies from controls (n=14) and patients suffering from Duchenne Muscular Dystrophy (DMD, n=23), **p=0.0092, unpaired t-test, data from GEO DataSets GSE6011. (B) NR1D1 expression in control or DMD primary myotubes, n=5-7. *p=0.0404 vs. control cells in panel B, unpaired t-test. (C) Representative curves and (D) peak fluorescence intensity of thapsigargin (TG)-induced Sarcoplasmic Reticulum (SR) Ca\(^ {2+}\) release in myoblasts from controls or patients suffering from Duchenne Muscular Dystrophy (DMD) treated with SR9009 (10µM) or vehicle. Cells are loaded with Fluo4-AM and SR Ca\(^ {2+}\) release is induced by the addition of 1µM TG. Results are expressed as means ± SEM of the Delta F/F0 ratio, n=3 controls, n=7 in both DMD groups. **p=0.0049 vs. control cells, unpaired t-test, $p=0.0138$ vs. DMD DMSO, paired t-test. (E) Hematoxylin and eosin and Sirius red staining of tibialis anterior muscles obtained from vehicle- and SR9009-injected mdx/Utr\(^{+/-}\) mice. Scale bars represent 100µm. (F) Myofiber cross-sectional area distribution (n=7-9), *p<0.05 vs. vehicle-treated mdx/Utr\(^{+/-}\) mice by 2-way ANOVA. (G) Circulating creatine phosphokinase (CPK) activity, n=8-9, *p=0.0329 vs. vehicle-treated mdx/Utr\(^{+/-}\) animals. (H) Muscular hydroxyproline, n=8-10, *p=0.0172. (I) Col1a2, (J) Pdgfra and (K) Mln gene expression; n=8-12, *p=0.0314, *p=0.0164 and *p=0.0402, respectively. (L) SERCA activity (n=8-10) in muscular microsomes from mdx/Utr\(^{+/-}\) mice treated for 20 days with SR9009 (100mg/kg) or vehicle; *p=0.0266. (M) In situ measurement of gastrocnemius-developed force (n=4-9, *p=0.0301).