The intracellular Ca\(^{2+}\) channel MCOLN1 is required for sarcolemma repair to prevent muscular dystrophy

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The integrity of the plasma membrane is maintained through an active repair process, especially in skeletal and cardiac muscle cells, in which contraction-induced mechanical damage frequently occurs in vivo\(^1\)\(^-\)\(^2\). Muscular dystrophies (MDs) are a group of muscle diseases characterized by skeletal muscle wasting and weakness\(^3\)\(^-\)\(^4\). An important cause of these group of diseases is defective repair of sarcolemmal injuries, which normally requires Ca\(^{2+}\) sensor proteins\(^5\)\(^-\)\(^8\) and Ca\(^{2+}\)-dependent delivery of intracellular vesicles to the sites of injury\(^8\)\(^-\)\(^9\). MCOLN1 (also known as TRPML1, ML1) is an endosomal and lysosomal Ca\(^{2+}\) channel whose human mutations cause mucolipidosis IV (ML4), a neurodegenerative disease with motor disabilities\(^10\)\(^-\)\(^11\). Here we report that ML1-mutations cause mucolipidosis IV (ML4), a neurodegenerative disease of skeletal muscle via Ca\(^{2+}\)-dependent vesicle exocytosis.

We used PCR genotyping to confirm in our targeted mouse strain the presence of a genetic deletion of Mcoln1 (referred to as ML1 KO)\(^11\) (Supplementary Fig. 1a; see Supplementary Data Set). Using reverse transcription PCR (RT-PCR), we detected no full-length ML1 transcript in skeletal muscle and cultured myoblasts isolated from ML1 KO mice (Fig. 1a). Consistent with these results, patch-clamping of the endolysosomal membranes\(^12\) showed that ML1-like currents (\(I_{ML1}\)) were activated in whole endolysosomes by ML-SA1, a membrane-permeable ML1-specific synthetic agonist\(^13\), in wild-type (WT) but not ML1 KO primary cultured myoblasts (Fig. 1b). \(I_{ML1}\) was potently inhibited by ML-SI compounds (Fig. 1b), which are membrane-permeable ML-specific synthetic inhibitors\(^14\).

At 1 month of age, ML1 KO mice are grossly healthy and do not show any obvious neurodegeneration\(^11\). However, when they are challenged with a 15° downhill treadmill test at the speed of 20 m/min, ML1 KO mice show a pronounced defect in their motor functions and a greatly reduced ability to remain on the treadmill (Fig. 1c). Histological analysis of various tissues involved in the movement impairment revealed, unexpectedly, that the skeletal muscles of ML1 KO mice had clear signs of dystrophy, even at 1 month of age (Fig. 1d,e). Indeed, by this early age, we detected individual necrotic and centrally nucleated fibers in ML1 KO skeletal muscle (Fig. 1d,e). In contrast, there was no obvious dystrophy in WT skeletal muscle at any age examined (Fig. 1e).

By 3 months of age, we commonly found central nucleation, fibrosis (fibrous scar tissue and fat replacement) and immune cell infiltration (Fig. 1d,e and Supplementary Fig. 1) in the skeletal muscles of ML1 KO mice. As observed in most animal models of MD\(^15\), the distribution of the dystrophic area in skeletal muscle was heterogeneous. For example, for the gastrocnemius muscle, the dystrophic area was mainly concentrated on the periphery of the muscle and the central region remained largely intact (Fig. 1d). A characteristic of MD is muscle regeneration triggered by degeneration, forming a cycle of degeneration and regeneration\(^4\)\(^-\)\(^9\). Hence, centrally nucleated muscle fibers and smaller-sized fibers are frequently observed, reflecting muscles undergoing active regeneration\(^4\)\(^-\)\(^9\). Consistent with this finding, ML1 KO fibers were relatively small, with a high degree of central nucleation (Fig. 1d,g,h).

ML1 KO mice exhibited progressive MD, with severity increasing with age (Fig. 1e–g). Muscle-specific heterogeneity is common in patients with MD, potentially resulting from the variability in use-dependent physical activity of different muscles\(^3\)\(^-\)\(^4\). In 1-month-old

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ML1 KO mice, only about half of the skeletal muscles, including the triceps, quadriceps, hamstring and gastrocnemius muscles, manifested dystrophy (Supplementary Fig. 1c). In contrast, the diaphragm, iliopecto, gluteus, soleus and tibialis anterior muscles appeared normal (Supplementary Fig. 1c). However, by 3 months of age, more skeletal muscles developed dystrophy (Supplementary Fig. 1d). Both type 1 slow-twitch and type 2 fast-twitch muscle fibers were dystrophic (Supplementary Fig. 2a). However, cardiac and smooth muscles in 3-month-old ML1 KO mice did not show obvious pathology (Supplementary Fig. 2b). These results suggest that ML1 KO mice exhibited early-onset, progressive and extensive MD.

Evans blue (EB) dye is a reliable in vivo marker of myofiber damage. A small but significant percentage of ML1 KO gastrocnemius myofibers were EB positive at rest (Fig. 2a,b). After a 15° downhill treadmill exercise, the percentage of EB-positive cells in the ML1 KO gastrocnemius muscle increased from 2% to 12% (Fig. 2a,b). In comparison, the percentage of EB-positive cells in WT littersmates never exceeded 1%, even after treadmill exercise (Fig. 2a,b).

Another measure of myofiber damage is the leakage of muscle proteins into the serum. Consistent with the EB analysis, the serum creatine kinase (CK) levels of ML1 KO mice were two- to three-fold higher than those of their WT littermates (Fig. 2c). Treadmill exercise increased serum CK levels further (Fig. 2c). These results suggest that an increase in muscle membrane damage underlies MD in ML1 KO mice.

Although muscle pathology and elevated CK levels were initially reported in some ML4 patients, ML4 has generally been considered a disease of neural degeneration, which could explain the motor defects of ML4 patients and ML1 KO mice. Consistent with previous reports, ML1 KO mice did exhibit neuronal cell death, but only at ages >5 months. At younger ages (1–3 months), motor neurons in the spinal cord did not show any obvious sign of neural degeneration.

Likewise, sciatic nerve myelination was normal in ML1 KO mice at 1 month of age (Fig. 2d). Furthermore, in conditions mimicking neural degeneration, such as sciatic nerve axotomy, we did not observe a dystrophic phenotype (Fig. 2e). Instead, axotomy resulted in muscle atrophy, which manifested as a homogeneous decrease in fiber size (Fig. 2f). In addition, gastrocnemius muscle from the mouse model of Fabry's disease showed denervation-like effects on the fiber size but not MD-like necrosis or central nucleation (Fig. 2f).

In 1-month-old ML1 KO mice, we saw only minimal evidence of lysosomal storage disease (LSD) in the dystrophic muscles (Supplementary Fig. 2e). These results suggest that muscle dystrophy in the ML1 KO mice is unlikely to be a secondary effect of neural degeneration or a lysosome storage defect.
To investigate directly the dystrophic mechanisms caused by ML1 deficiency, we performed a rescue experiment involving intramuscular injection of an adeno-associated virus (AAV) carrying the GFP-ML1 transgene (AAV-GFP-ML1), which typically infected most (>85%) of the muscle fibers. As observed in many lysosomal storage disorders (LSDs), ML1 KO muscle showed a compensatory increase in the key lysosomal protein Lamp1 (ref. 13), as indicated by Lamp1 immunofluorescence staining and western blot analysis (Supplementary Fig. 3a,b).

However, when compared with the uninfected adjacent and contralateral muscle fibers, ML1 KO gastrocnemius muscle infected with AAV-GFP-ML1 (localized in Lamp1-positive compartments; Supplementary Fig. 3c,d) showed a strong AAV infection–mediated decrease of elevated Lamp1 expression (Fig. 2g and Supplementary Fig. 3e). AAV-ML1-GFP infection reduced the dystrophic area (Fig. 2h) and collagen content (Fig. 2i) of the muscle as well as the percentage of EB-positive muscle fibers (Fig. 2j). Hence, expression of ML1 in muscle was sufficient to rescue the MD of ML1 KO mice, suggesting a cell-autonomous mechanism as the underlying cause of MD in these mice.

Mechanical stress can cause myofiber necrosis by two separate mechanisms. First, the sarcolemma of a muscle fiber could be more susceptible to damage, as seen in dystrophin (a core component of the dystrophin-glycoprotein complex (DGC)) mutant (mdx) mice (4). Second, a muscle fiber could have a defect in sarcolemma repair, as seen in dysferlin or MG53 knockout mice (5,9,22). Most human MD mutations are linked to defects in the components of the DGC. However, we saw no obvious decrease in expression of any of the core or accessory components of the DGC that we examined, which included dystrophin, β-dystroglycan (β-DG), integrin β1 and laminin (Fig. 3a,b). Furthermore, the expression of dysferlin, caveolin 3 and MG53, three proteins known to be involved in sarcolemma repair and human MD (5,23), also showed no decrease in ML1 KO muscle (Fig. 3a,b).

To create plasma membrane disruptions and to evaluate the resealing efficiency, we irradiated single myofibers isolated from the flexor digitorum brevis (FDB) muscle of WT and ML1 KO mice using a two-photon laser (9). FM1-43, a membrane-impermeable fluorescent dye that preferentially adheres to lipids, is commonly used to detect membrane disruptions (5). We observed rapid entry and accumulation...
of FM1-43 dye within seconds after laser irradiation (Fig. 3c and Supplementary Fig. 4a). However, in WT muscle fibers, dye entry ceased shortly (1–2 min) after irradiation, suggesting successful membrane resealing (Fig. 3c). In contrast, upon identical laser irradiation of fibers from ML1 KO mice, uptake of the FM1-43 dye continued at the injury sites for several minutes (Fig. 3c), suggesting failed membrane resealing. Removal of extracellular Ca\(^{2+}\) caused rapid accumulation of FM1-43 dye at the injury sites, which was indistinguishable for myofibers from WT and ML1 KO mice (Supplementary Fig. 4b). We also observed defective membrane resealing in myotubes from ML1 KO mice that were exposed to mechanical damage elicited by microelectrode penetration into the sarcolemmal membrane (Fig. 3d). Unlike most myotubes from WT mice, most (>80%) of those from ML1 KO mice could not ‘survive’ the prolonged contractions caused by continuous Ca\(^{2+}\) entry.

Next, we performed pharmacological experiments on myotubes derived from the C2C12 mouse cell line using the microelectrode penetration assay\(^ {24}\). We did not see substantial uptake of FM4-64 dye (a red-colored analog of the FM1-43 dye) at injury sites, even with repeated penetrations (Fig. 3e). In contrast, removal of extracellular Ca\(^{2+}\) substantially increased the entry of FM dyes (Supplementary Fig. 4c), suggesting that the influx of extracellular Ca\(^{2+}\) is essential for repair\(^ {8}\). In the presence of BAPTA-AM, used to chelate intracellular Ca\(^{2+}\), or glycyl-l-phenylalanine 2-naphthylamide (GPN), a lysosome-targeted cathepsin C substrate, to specifically deplete the lysosomal Ca\(^{2+}\) store\(^ {13}\), we observed substantial dye entry even under normal extracellular Ca\(^{2+}\) concentrations (2 mM; Fig. 3e). These results suggest that lysosomal Ca\(^{2+}\) also has a role in membrane resealing.

To monitor the Ca\(^{2+}\) levels at injury sites, we transfected myoblasts from WT and ML1 KO mice and C2C12 cells with Lamp1-GCaMP3,
a lysosome-targeted genetically encoded Ca\textsuperscript{2+} indicator\textsuperscript{13}. We observed transient Ca\textsuperscript{2+} increases at injury sites in the presence or absence of external Ca\textsuperscript{2+} (Supplementary Fig. 4d–g). However, release of intracellular Ca\textsuperscript{2+} was less in myoblasts from ML1 KO mice or in the presence of ML-SI compounds\textsuperscript{14} (Supplementary Fig. 4f,g), suggesting a contribution to Ca\textsuperscript{2+} release from lysosomes during membrane damage or the early stage of membrane repair. In the presence of external Ca\textsuperscript{2+}, we observed a prolonged Ca\textsuperscript{2+} influx in myoblasts from ML1 KO mice or ML-SI3-treated C2C12 myoblasts (Supplementary Fig. 4d,e). Hence, ML1 has a dual role in promoting the initial phase of Ca\textsuperscript{2+} increase (within seconds) but inhibiting the prolonged phase of Ca\textsuperscript{2+} increase (lasting minutes).

To test whether ML1 has a direct role in membrane repair, we acutely inhibited ML1 channel function using ML-SIs. Notably, in experiments performed by researchers who were blind to experimental conditions, substantial FM4-64 dye uptake was seen in the presence of three structurally independent ML-SI compounds (Fig. 3e and Supplementary Fig. 4c). We also used ML1 inhibitors to test the role of ML1 in sarcolemma repair in vivo. Cardiotoxin VII4 (CTX) is a toxin that can induce membrane damage in vivo to cause EB dye accumulation in muscle cells\textsuperscript{24}. EB-positive muscle cells were more numerous in CTX-treated muscle from ML1 KO mice than in muscle from CTX-treated WT control mice (Supplementary Fig. 4h). Notably, co-injection of ML-SI3 with CTX markedly increased the percentage of EB-positive muscles in CTX-treated WT mice, to the same level as in CTX-treated ML1 KO mice (Fig. 3f and Supplementary Fig. 4h).

Chemical injuries from streptolysin O (SLO) toxin is often used to induce membrane repair responses, and propidium iodide (PI) staining is a common readout for membrane damage and cell viability\textsuperscript{25}. In non-muscle cells, including mouse embryonic fibroblasts (MEFs) and bone marrow–derived macrophages (BMMs), treatment with SLO resulted in more PI-positive cells from ML1 KO mice than from WT mice (Fig. 4a and Supplementary Fig. 5). In addition, ML-SI3 increased PI staining in WT, but not knockout cells (Fig. 4a and Supplementary Fig. 5). Conversely, ML1 overexpression decreased PI staining (Supplementary Fig. 5). These results suggest that ML1 may be a core component of the membrane repair machinery in both muscle and non-muscle cells.

Membrane resealing requires the fusion and exocytosis of intracellular vesicles at sarcolemma injury sites\textsuperscript{8}. Upon SLO treatment, pHluorin total internal reflection fluorescence (TIRF) imaging\textsuperscript{26} showed that ML1 and Vamp7 doubly-positive vesicles underwent exocytosis (Supplementary Fig. 6a and Supplementary Video 1). Exocytosis of ML1-resident vesicles may lead to the appearance of ML1 proteins at the plasma membrane. Hence, measurement of whole-cell ML1 currents may provide a readout for exocytosis\textsuperscript{4,8}. Consistently, SLO treatment also increased whole-cell I\textsubscript{ML1} by two-fold to three-fold in C2C12 cells (Fig. 4b,c). In contrast, ML-SI3–mediated inhibition of ML1, or knockout of ML1, substantially reduced SLO-induced lysosomal exocytosis as measured by Lamp1 surface staining\textsuperscript{27} (Supplementary Fig. 6b,c) and release of lysosomal enzymes (acid phosphatase, AP, or acid sphingomyelase, acid SMase\textsuperscript{28}) (Fig. 4d,e) in muscle cells and MEFs. Collectively, these results suggest that ML1-mediated lysosomal exocytosis has an important role in membrane resealing.

Consistent with the possibility that overexpression of ML1 promotes membrane repair in mdx muscle\textsuperscript{21}. EB dye uptake and dystrophic area were both substantially less in AAV-GFP-ML1–infected muscle than in the contralateral noninjected control muscle of mdx mice(Fig. 4f,g).

ML1 KO mice exhibit a primary early-onset MD that is caused by defective membrane resealing. A similar but less dramatic dystrophic phenotype is observed in mice lacking MG53 or dysferlin, two proteins known to be involved in membrane repair\textsuperscript{5,9}. By interacting with Ca\textsuperscript{2+} sensors in the vesicles, for example, dysferlin and Syt-VII\textsuperscript{15,9}, ML1 may mediate membrane repair by promoting vesicle exocytosis in both muscle and non-muscle cells (Supplementary Fig. 7). The initial rapid Ca\textsuperscript{2+} increase, in 10–20 s, is essential for triggering the resealing process. Depending on the nature of the damage and the size...
of the wound, three different mechanisms may be used for membrane repair: membrane patching, endocytosis and shedding\textsuperscript{4,28,29}. Previous studies have unequivocally established the roles of extracellular Ca\textsuperscript{2+} in all \textit{in vitro} assays and models of membrane repair\textsuperscript{5,27,29}. We show here that lysosomal Ca\textsuperscript{2+} is also essential for this process, even in the presence of extracellular Ca\textsuperscript{2+}, suggesting an involvement of more than one Ca\textsuperscript{2+} source. Consistent with this idea, multiple Ca\textsuperscript{2+} sensors are implicated in sarcolemma repair, including dysferlin, myoferlin, annexin, Syt-VII, Alix/ALG-2 and calcineurin\textsuperscript{8,29}. Although lysosomes have long been implicated in membrane repair in non-muscle cells\textsuperscript{27}, their role in sarcolemma repair has remained unclear. Notably, expression of the housekeeping lysosomal protein Lamp1, which is compensatorily upregulated in many LSDs, is also elevated in dysferlin-null mice\textsuperscript{116}. In addition, mice lacking Syt-VII, a lysosome-specific Ca\textsuperscript{2+} sensor, exhibit a MD-like phenotype\textsuperscript{8}. Hence, the sarcolemma repair system may have a close relationship with the late endocytic pathway.

**METHODS**

Methods and any associated references are available in the online version of the paper. 

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

X.C. initiated the project; H.X., X.C., J.D. and R.H. designed the research; X.C., X.Z., Q.G., M.A.S., M.A., W.L.T., Y.Z. and I.D. performed the research; N.S., X.L., A.G.G., X.W., M.F. and L.X. contributed the new reagents; X.C., X.Z., Q.G., M.A.S., M.A., W.L.T., J.D., R.H. and H.X. analyzed the data; H.X. and X.C. wrote the paper with input from all the authors.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Reverse transcription-PCR. Total RNA was extracted from muscle tissues or cultured myoblasts and dissolved in TRizol (Invitrogen). First-strand cDNA, synthesized with Superscript III RT (Invitrogen), was used for RT-PCR analysis of ML1 expression based on the following intron-spanning primer pair (and L32 control). ML1: forward, 5′-AACACCCCGA GTGTCCCGAG-3′; reverse, 5′-GAATGACCC GACCAGACT-3′. L32: forward, 5′-TGGTGAAGCG CAAGATCGTC-3′; reverse, 5′-CTTCTCC GCA CCGTGTGTGC-3′.

Plasmid construction. Generation of the plasmids used in this study (GFP-ML1, mCherry-ML1, GCaMP3-ML1, Lamp1-GCaMP3 and Vamp7-pHluorin) was reported previously. Briefly, cells were treated with 1 µM vacuolin-1 for 2–5 h to increase the size of endosomes and lysosomes. Whole-endosome recordings were performed on isolated enlarged endosomes. The bath (internal/cytoplasmic) solution contained 140 mM K-gluc, 4 mM NaN, 1 mM EGTA, 2 mM Na₂ATP, 2 mM MgCl₂, 0.39 mM CaCl₂, 0.2 mM GTP and 10 mM HEPES (pH adjusted with KOH to 7.2; the free [Ca²⁺] was estimated to be ~100 nM on Maxchelator software (http://maxchelator.stanford.edu/)). The pipette (luminal) solution consisted of a ‘low-pH Tyrode’s solution’ with 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM MES and 10 mM glucose (pH 4.6). All bath solutions were applied via a perfusion system to achieve a complete solution exchange within a few seconds. Data were collected with an Axopatch 2A patch clamp amplifier, Digidata 1440 and pClamp 10.0 software (Axon Instruments). Currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21–23 °C), and all recordings were analyzed with pClamp 10.0 and Origin 8.0 (OriginLab).

Whole-cell electrophysiology. Whole-cell recordings were performed as described. Briefly, single FDB fibers were mounted on a heated stage on a spinning disk confocal imaging system (Amersham Pharmacia Biotech).

Mouse lines. The generation and characterization of ML1 KO mice (in a B6;129 mixed genetic background) were as described. mkd mice were ordered from Jackson Laboratories. Faby’s mice were a gift from J. Shayman (University of Michigan). Mice were used under approved animal protocols and the Institutional Animal Care Guidelines at the University of Michigan. WT littersmates were used as controls in the mouse experiments. No statistical method was used to predetermine sample size. Mice randomized as to sex were randomly assigned to both control and testing groups, each typically containing 3–5 animals.

Treadmill exercise. Mice were trained to run on an Exer-6M treadmill (Columbus Instruments) with a 15° downhill angle at the speed of 12–20 m/min.

Muscle cell culture. Mouse myoblasts were prepared and cultured as described. Briefly, limb muscles were isolated and dissociated with 1% collagenase and 0.25% trypsin treatment at 37 °C. After preplating on a standard Petri dish (non-tissue culture coated) for 60–90 min to remove fibroblasts, muscle cells were maintained at 37 °C and 5% CO₂ in F10 medium with 20% FBS (Gibco). To induce differentiation, myoblasts were grown to confluence before switching to DMEM containing 5% horse serum. For transient transfections, myoblasts were plated at 70% confluence and transfected with 20% FBS (Gibco). Live-cell imaging experiments were performed on isolated enlarged endosomes. The bath (internal/cytoplasmic) solution contained 140 mM K-gluc, 4 mM NaN, 1 mM EGTA, 2 mM Na₂ATP, 2 mM MgCl₂, 0.39 mM CaCl₂, 0.2 mM GTP and 10 mM HEPES (pH adjusted with KOH to 7.2; the free [Ca²⁺] was estimated to be ~100 nM on Maxchelator software (http://maxchelator.stanford.edu/)). The pipette (luminal) solution consisted of a ‘low-pH Tyrode’s solution’ with 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM MES and 10 mM glucose (pH 4.6). All bath solutions were applied via a perfusion system to achieve a complete solution exchange within a few seconds. Data were collected with an Axopatch 2A patch clamp amplifier, Digidata 1440 and pClamp 10.0 software (Axon Instruments). Currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21–23 °C), and all recordings were analyzed with pClamp 10.0 and Origin 8.0 (OriginLab).

Whole-cell electrophysiology. Whole-cell recordings were performed as described. Briefly, single FDB fibers were mounted on a glass bottom chamber in Tyrode’s or zero Ca²⁺ solution in the presence of 2.5 µM green-colored FM1-43 dye (Molecular Probes). To induce damage to the muscle fibers, a selected region (5 µm × 5 µm) of the plasma membrane was irradiated for 5 s with a two-photon laser (laser power 3700 W at wavelength 820 nm) in a Leica Fluoview 300 confocal microscope system. Images were captured at 10-s intervals. For every image taken, the fluorescence intensity at the site of the damage was measured with ImageJ software. Fibers that had defective membrane resealing showed dye accumulation at the injury sites

H&E and histochemical staining. H&E staining was performed in 12-µm sections that were prepared from freshly frozen tissues and fixed with 4% PFA. Myosin ATPase staining was performed at pH 4.3; at this pH, type I slow-twitch fibers are dark-colored and type 2 fast-twitch fibers are light-colored.

Collagen content measurement. The level of hydroxyproline, a major component of collagen, was measured using the Hydroxyproline Assay Kit (Sigma).

Sciatic axotomy. Right sciatic nerves of 3-month-old WT mice were exposed, and a 5-mm segment of the nerve was removed surgically. The wound was closed with silk sutures. The gastrocnemius muscle was collected for H&E staining 2 weeks after the axotomy.

Myofiber damage assay. After mice were sacrificed by cervical dislocation, FDB muscles were surgically removed to be digested in a Tyrode’s solution containing type I collagenase (2 mg/ml; Sigma) at 37 °C for 60 min. After resuspension in the Tyrode’s solution, single FDB fibers were mounted on a glass bottom chamber in Tyrode’s or zero Ca²⁺ solution in the presence of 2.5 µM green-colored FM1-43 dye (Molecular Probes). To induce damage to the muscle fibers, a selected region (5 µm × 5 µm) of the plasma membrane was irradiated for 5 s with a two-photon laser (laser power 3700 W at wavelength 820 nm) in a Leica Fluoview 300 confocal microscope system. Images were captured at 10-s intervals. For every image taken, the fluorescence intensity at the site of the damage was measured with ImageJ software. Fibers that had defective membrane resealing showed dye accumulation at the injury sites
throughout the time course of the experiment, whereas, for resealed fibers, dye influx stopped typically within 1 min.

**Microelectrode penetration damage.** C2C12 myoblasts or primary myoblasts were allowed to differentiate into myotubes for 3–5 d before use. Microelectrodes (borosilicate glass capillaries; World Precision Instruments) were connected to a three-axis micromanipulator (MHH-103 Narishige International) to precisely control the penetration of the cell membrane in the presence of 2.5 µM red-colored FM4-64 dye (Molecular Probes). After a microelectrode was gently attached to a myotube, a further displacement of ~2 µm was considered penetration. Images were taken on an Olympus microscope equipped with a QIClick digital CCD Camera (QImaging). Live imaging of myoblasts was performed on a heated stage with the spinning disk confocal imaging system described above. Microelectrodes attached to the three-axis micromanipulator MP-285 (Sutter Instruments) were used to induce penetration. The investigator was blinded to allocation during experiments and outcome assessment.

**Evans blue dye uptake.** EB dye (1%; 10 ml per kg body weight; Sigma) was injected into the intraperitoneal space of 1-month-old mice 8–16 h before tissue collection. Dissected gastrocnemius muscles were frozen for cryosectioning.

**Flow cytometry PI staining.** His-tagged SLO (carrying a cysteine deletion that eliminates the need for thiol activation25) was provided by R. Tweeten (University of Oklahoma) and purified using Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) in a BL21 (University of Oklahoma) and purified using Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) in a BL21 E. coli expression system. The protein concentration was measured using the Bradford assay, and the aliquots of proteins were stored at −80 °C until use. For each experiment, about 2 × 10^6 cells were trypsinized, washed with Tyrode's solution and incubated with SLO (2–5 µg/ml) for 10 min at 37 °C. Titration of SLO was performed to determine the minimum concentration required for cell permeabilization in 0 Ca^2+ and the maximum concentration not causing significant cell loss. SLO-treated cells were then stained with 200 µg/ml propidium iodide (PI; Sigma-Aldrich) for 5 min and analyzed by FACS Flow Cytometry (iCyt Synergy; Sony). More than 10,000 cells per experiment were analyzed for forward-angle scatter, right-angle scatter and fluorescence intensity.

**PHluorin total internal reflection fluorescence imaging.** TIRF imaging was performed at 37 °C using a VisiTech Infinity three array-scanning confocal microscope (VisiTech International Ltd.) equipped with a 100× APO TIRF objective oil-immersion (Nikon, NA 1.49). MetaMorph v7.71 image acquisition software was used to process the data.

**Electron microscopy analysis.** For EM analysis, animals were perfused with 4% PFA and 2.5% glutaraldehyde in PBS. Muscles were sliced and intact tendons. Samples were prepared with standard embedding and sectioning procedures.

**AAV generation and infection.** CAG promoter-driven AAV1/2-GFP-ML1 virus was generated by the Gene Transfer Vector Core of the University of Iowa. Virus injection was performed on one side of the gastrocnemius muscle of 1-month-old ML1 KO or 10-day-old mdx mice. The other side of the gastrocnemius muscle was used as the contralateral control. Injected and noninjected contralateral leg muscles were examined after 4 weeks.

**Data analysis.** Data are presented as the mean ± s.e.m. Statistical comparisons were performed with analysis of variance (ANOVA). A P value <0.05 was considered statistically significant.

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