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Distinct Roles for Laminin Globular Domains in Laminin α1 Chain Mediated Rescue of Murine Laminin α2 Chain Deficiency

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Abstract

Background: Laminin α2 chain mutations cause congenital muscular dystrophy with dysmyelination neuropathy (MDC1A). Previously, we demonstrated that laminin α1 chain ameliorates the disease in mice. Dystroglycan and integrins are major laminin receptors. Unlike laminin α2 chain, α1 chain binds the receptors by separate domains; laminin globular (LG) domains 4 and LG1-3, respectively. Thus, the laminin α1 chain is an excellent tool to distingush between the roles of dystroglycan and integrins in the neuromuscular system.

Methodology/Principal Findings: Here, we provide insights into the functions of laminin α1LG domains and the division of their roles in MDC1A pathogenesis and rescue. Overexpression of laminin α1 chain that lacks the dystroglycan binding LG4-5 domains in α2 chain deficient mice resulted in prolonged hifespan and improved health. Importantly, diaphragm and heart muscles were corrected, whereas limb muscles were dystrophic, indicating that different muscles have different requirements for LG4-5 domains. Furthermore, the regenerative capacity of the skeletal muscle did not depend on laminin α1LG4-5. However, this domain was crucial for preventing apoptosis in limb muscles, essential for myelination in peripheral nerve and important for basement membrane assembly.

Conclusions/Significance: These results show that laminin α1LG domains and consequently their receptors have disparate functions in the neuromuscular system. Understanding these interactions could contribute to design and optimization of future medical treatment for MDC1A patients.


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Introduction

Congenital muscular dystrophy type 1A (MDC1A) is an autosomal recessive disorder caused by mutations in the gene encoding laminin (LM) α2 chain. The general clinical hallmarks of MDC1A include neonatal onset of muscle weakness, hypotonia often associated with joint contractures, inability to stand and walk, elevated levels of creatine kinase, white matter abnormalities and dysmyelination neuropathy. Histological changes of muscles comprise fiber size variability, massive degeneration and extensive connective tissue infiltration. Most patients die as teenagers since there is no treatment for this devastating disease [1]. Several mouse models for MDC1A exist (e.g. generated LMα2 chain mutants dy/dyC and dy/dyS and the spontaneous mutant mouse strain dy/dyS) and they adequately mirror the human condition [2–4].

LMs are extracellular proteins formed by α, β and γ chains. Together with other extracellular matrix components LMs form specialized extracellular matrices called basement membranes [5]. LM-211 (composed of α2, β1 and γ1 chains) is the major LM isoform expressed in muscle and peripheral nerve. Through interaction with transmembrane receptors it regulates major functions of the neuromuscular system and provides structural support to muscle fibers [6]. In muscle, at least two distinct protein complexes are known to be the key receptors for LMα2 chain; dystroglycan and integrin α7β1. Their importance is underscored by the fact that absence of integrin α7 chain, as well as hypoglycosylation of α7-dystroglycan cause various forms of congenital muscular dystrophy [7,8]. Furthermore, different studies involving manipulation of the dystroglycan gene in mice revealed an important role for dystroglycan in skeletal muscle [9–11]. Several studies indicated that the function of integrin α7 subunit and dystroglycan, being a part of the dystrophin-glycoprotein complex, could overlap [12–14]. However, recent studies show that whereas both dystroglycan and integrin α7 chain contribute to force-production of muscles, only dystroglycan contributes to the preservation of sarcolemmal integrity [15].

LMα2 chain receptors present in peripheral nerve include dystroglycan, integrins α6β1, α7β1 and possibly integrin α6β4 [16,17]. Dystroglycan, β1 and β4 integrin subunits have been
shown to be important for different aspects of myelination and morphology of peripheral nerves, as revealed by conditional disruption of their genes in Schwann cells [10–20]. Thus, LM-211 is a central player linking these receptors and their functions in the neuromuscular system.

LMα1 chain also binds to dystroglycan, integrin αβ1 and integrin α7β1 (and perhaps integrin α6β4) [17,21–24]. Yet, it is not expressed in the neuromuscular system [25]. We have previously explored the possibilities of paralogous gene therapy for MDC1A and demonstrated that LMα1 chain is an excellent substitute for LMα2 chain in murine muscle, peripheral nerve and tests [25–28]. Additionally, LMα2 chain deficiency leads to perturbed expression of integrin αβ7 unit, and reduced expression of the core protein of α-dystroglycan (but not α-dystroglycan glycosylation), at the sarcolemma [29–31]. Notably, LMα1 chain overexpression restores integrin α7 chain expression, indicating that this receptor could be crucial for improvement of muscle function in dystrophic animals [32].

The LMα1 and α2 chains bind dystroglycan and integrins by distinct domains. The α1 chain binds dystroglycan via its C-terminal LG4 domain and integrin binding occurs via α1 LG1-3 [33,34]. This is different from LMα2 chain binding where there is considerable overlap in binding to dystroglycan and integrins. Both α2LG4-5 and α2LG1-3 bind dystroglycan, whereas only α2LG1-3 binds integrins [23,35]. The LMα1 chain can thus be used more efficiently to distinguish between the roles of LM binding to dystroglycan and integrins in the neuromuscular system. Since LMα1 chain functions almost equally well as α2 chain in the neuromuscular system, we used this subunit in order to dissect the roles of αLG domains and their receptors in MDC1A pathogenesis and rescue. Hence, we produced and characterized animals completely deficient in LMα1 chain (dy3K/dy3K mice) that lacks the dystroglycan binding site (LG4-5 domains at the C-terminus, also known as the E3 fragment), but retains the integrin binding site (LG1-3, see Fig. 1A) [33,34].

Materials and Methods

Ethics statement

All mouse experimentation was approved by the local (Lund district) ethics committee (permit number M62-09). All mice were maintained in animal facilities according to animal care guidelines.

Transgenic construct

Approximately 1 kb of the C-terminal part was removed from mouse full-length LMα1 chain cDNA to generate truncated cDNA (δE3LMα1). An in frame deletion between nucleotides 8248–9289 (corresponding to LG4-5 domains) was accomplished by DraIII-SmaI restriction cutting and fusion of an XhoI site with a BglII site. This DNA was cloned into the pCAGGS vector [23], containing a CMV enhancer and a β-actin promoter.

Transgenic animals

Transgenic mice were generated by microinjections of transgene DNA into the pronucleus of fertilized single-cell C57BL/BL/CBA embryos (Lund Transgenic Core Facility, Lund University, Sweden). Mice carrying δE3LMα1 chain DNA were identified by PCR as described previously [25]. Positive founders overexpressing truncated LMα1 chain in the neuromuscular system (lines No. 3 and 4) were further bred with dy3K/+ mice [2], followed by sib breeding to generate LMα2 chain deficient animals that express δE3LMα1 chain (dy3K/δE3 mice). Dy3K/dy3K mice overexpressing full length LMα1 chain (dy3KLMα1 mice) were previously described [25–28]. Dy/dy mice used for heart studies were obtained from Jackson Laboratory.

Exploratory locomotion and body and muscle weight analyses

Exploratory locomotion was examined in an open field test. A mouse was placed into a new cage and allowed to explore the cage for 5 min. The time that the mouse spent moving around was measured. For all experiments, 10-week-old dy3K/δE3 animals (n = 16) were compared with 10-week-old control mice (wild-type or dy3K/+ [n = 8] and 5-week-old dy3K/dy3K mice (n = 6). For weight analysis dy3K/δE3, control mice and dy3K/dy3K animals were sex- and age-matched (5-week-old) (n = 14, n = 3, n = 11, respectively, for females; n = 8, n = 4, n = 8, respectively, for males). Quadriceps and tibialis anterior muscles from 2-month-old wild-type [n = 5], dy3K/δE3 (n = 3) and 4-week-old dy3K/dy3K mice (n = 4) were used to estimate the ratio of wet muscle weight to body weight. Muscles from both legs were weighed and average muscle mass was calculated. Unpaired t-test was used for statistical analysis.

Creatine kinase activity

Blood was collected from the tail vein of 2-month-old control mice (wild-type or dy3K/+ (n = 10). dy3K/δE3 (n = 10) and 4-week-old dy3K/dy3K mice (n = 3)) into EDTA-tubes and spun down two times for 5 minutes at 3500 rpm. CK_P_S_cobas method was used by Clinical Chemistry Laboratory at Skåne University

Figure 1. Generation of δE3LMα1 transgenic animals. (A) Scheme presenting LM-111 structure. Full-length LMα1 chain with LG1-5 domains and truncated LMα1 chain (δE3LMα1) with LG1-3 domains are marked together with their transmembrane receptors. (B) Schematic presentation of transgenic construct with denoted 1 kb deletion (LG4-5). Restriction sites used to engineer the construct are shown.

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Hospital to quantify enzyme activity in plasma. Unpaired t-test was used for statistical analyses.

**Histology and immunofluorescence microscopy**

Skeletal muscle, heart, peripheral nerve and spinal roots cryosections (7 μm) from control (wild-type or dy3K/+), dy3K/dy3K, dy/dy, dy3K/δE3 and dy3KLMα1 mice were either stained with hematoxylin and cosin or subjected to immunofluorescence analysis using following antibodies: rat monoclonal mAb200 against LMα1LMG4 domain [25], rabbit polyclonal 1057+ against LMα1LN/La domain (N-terminus) (kindly provided by Dr. T. Sasaki) [36], rabbit polyclonal 1100+ against LMα1 (kindly provided by Dr. T. Sasaki), rabbit polyclonal 1113+ against LMα5 (kindly provided by Dr. T. Sasaki), rat monoclonal MTn15 against tenascin-C [25], rabbit polyclonal anti-collagen, type IV (Chemicon), mouse monoclonal F1.652 against embryonic myosin heavy chain (Developmental Studies Hybridoma Bank, Iowa), rabbit polyclonal anti-collagen, type IV (Chemicon), mouse monoclonal 46 against caspase-3 (BD Transduction Laboratories), mouse on mouse kit (Vector) was used for staining mouse monoclonal IIH6 against α7 integrin and mouse monoclonal 4 against caspase-3 (BD Transduction Laboratories), rabbit polyclonal U31 against LMα1, rabbit polyclonal 1057 (kindly provided by Dr. T. Sasaki), rabbit polyclonal 1057 (kindly provided by Dr. T. Sasaki), rabbit polyclonal anti-collagen IV (Chemicon), mouse monoclonal 1113+ against LMα5 (kindly provided by Dr. T. Sasaki), rabbit polyclonal anti-collagen IV (Chemicon), mouse monoclonal 46 against caspase-3 (BD Transduction Laboratories), Mouse on mouse kit (Vector) was used for staining with embryonic myosin heavy chain according to manufacturer instructions. Tissues were fixed with 4% PFA at room temperature (for laminin, tenascin-C, embryonic myosin heavy chain, collagen-IV and caspase-3 stainings), or with acetone at 2°C for 10 min, washed and mounted with FluorSave (Calbiochem). By fluorescence microscopy analysis, EBD uptake into muscle fibers was visualized by red emission.

**Immunoblotting**

For LM detection proteins were isolated from 100 mg of dy3K/δE3 and dy3KLMα1 muscles (3 mice from each group) by brief sonication in 1 mmol/L EDTA in TBS with 1:25 dilution of Protease Cocktail (Pierce). Dystroglycan was isolated using agarose dy3K/E3 and dy3K/dy3K, mouse monoclonal IIH6 against α7-dystroglycan (Upstate Biotechnology), mouse monoclonal F1.652 against embryonic myosin heavy chain (Developmental Studies Hybridoma Bank, Iowa), rabbit polyclonal anti-collagen, type IV (Chemicon), mouse monoclonal 46 against caspase-3 (BD Transduction Laboratories). Mouse on mouse kit (Vector) was used for staining with embryonic myosin heavy chain according to manufacturer instructions. Tissues were fixed with 4% PFA at room temperature (for laminin, tenascin-C, embryonic myosin heavy chain, collagen-IV and caspase-3 stainings), or with acetone at −20°C (for integrin α7B) or with 8% formaldehyde, followed by methanol at −20°C (for α-dystroglycan). Sections were analyzed using a Zeiss Axioplan fluorescence microscope. Images were captured using an ORCA 1394 ER digital camera with Openlab 3 software. Images were prepared for publication using Adobe Photoshop software.

**Quantification of fiber size distribution, central nucleation and fiber number**

Diaphragm and limb muscles from at least 3 animals from each group (4–6-week-old wild-type, dy3K/dy3K and dy3K/δE3 mice) were analyzed. Minimal Feret’s diameter was measured [38] for at least 2600 fibers for each group. The same number of fibers was used for quantification of fibers with centrally located nuclei. An additional group of 4–6-month-old dy3K/δE3 animals was included for quantification of diaphragm fibers. Fibers from quadriceps muscle from 4–6-week-old wild-type (n = 3), dy3K/dy3K (n = 3) and dy3K/δE3 mice (n = 3) were counted within a square of 64 x 106 pixels2. Unpaired t-test was used for statistical analysis.

**Treadmill exercise and Evans blue dye injection**

Dy3K/δE3 mice (n = 4) were exercised for 30 min on a treadmill Exer 6M (Columbus Instruments) at a downhill angle of 15°. During the first 2 min the speed was gradually increased from 7 m/min up to 14–16 m/min. Within 30 min after completed exercise the mice were injected i.p. with Evans blue dye (EBD) (Sigma Aldrich) dissolved in sterile saline (concentration: 0.5 mg EBD/0.05 ml saline; amount: 50 μl per 10 g body weight). After approximately 24 h, muscles were collected and quickly frozen in liquid nitrogen. Unexercised mice were injected with EBD and used as controls. Muscle cryosections (8 μm) were fixed in ice-cold acetone at −20°C for 10 min, washed and mounted with FluorSave (Calbiochem). By fluorescence microscopy analysis, EBD uptake into muscle fibers was visualized by red emission.

**Cardiotoxin injections**

Tibialis anterior muscles from 6 control (wild-type or dy3K/+), 6 dy3K/dy3K and 6 dy3K/δE3 mice were injected with cardiotoxin (10 μmol/L in saline). Control and dy3K/δE3 mice were 2–3-month-old. Dy3K/dy3K mice were 3-week-old. Three mice from each group were sacrificed 4 days after injection and the other 3 after 11 days. Both injected and contralateral uninjected tibialis anterior muscles were collected and analyzed.

**Electron microscopy and toluidine blue staining**

Quadriceps femoris muscles, heart, diaphragm, sciatic nerves and spinal roots from wild-type, dy3K/dy3K and dy3K/δE3 mice were fixed for 2 hours with 1.5% glutaraldehyde/1.5% paraformaldehyde (Sigma) as described before [32]. Lysates containing LM, integrin and dystroglycan were separated on 5% or 8% polyacrylamide-SDS gels under reducing or non-reducing conditions. EHS LM (Invitrogen) was used as a control for LM blotting.

**Results**

**Generation of dy3K/dy3K mice overexpressing δE3LMα1 chain**

We have generated mice overexpressing LMα1 chain devoid of LG4-5 domains (comprising the E3 fragment) under the control of a CMV enhancer and β-actin promoter (Fig. 1A and B) (δE3 mice), Mice overexpressing δE3LMα1 in skeletal muscle, peripheral nerve and heart were maintained (transgenic lines No. 3 and 4) (Figure S1, see also Fig. 2). The expression of truncated LMα1 chain was detected using antibodies against the N-terminal domains of LMα1 chain and the LG4 domain, respectively. Immunofluorescence staining with the antibody directed against N-terminal domains of LMα1 chain demonstrated patchy expression of truncated LMα1 chain in basement membranes of skeletal and cardiac muscle, and in endoneurium and perineurium

![Image](https://example.com/image.png)
Laminin receptors in MDC1A

A

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B

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- Lα1
- α-actinin

Bar chart showing Lα1 and α-actinin levels with * indicating significance.
of sciatic nerve of ΔE3 transgenic mice (Figure S1). No staining was detected with the antibody directed towards LG4 domain, indicating the overexpression of truncated LMα1 chain. Staining with both antibodies was detected in LMα1TG mice overexpressing full-length LMα1 chain (Figure S1) (described in 25) and indicated a higher level and more homogenous expression of LMα1 chain in these animals. Notably, overexpression of truncated LMα1 chain in mice revealed no discernible pathological phenotypes.

Next, ΔE3 mice from line 3 and 4 were further mated with mice carrying the mutation in Lama2 gene (dy3K/+), to create mice that are devoid of LMα2 chain but instead overexpress ΔE3LMα1 chain (dy3K/ΔE3 mice).

Expression of truncated LMα1 chain is upregulated upon LMα2 chain deficiency

We analyzed the expression of ΔE3LMα1 chain in dy3K/ΔE3 mice in a similar manner as in ΔE3 mice (only the staining with the antibody against N-terminal domains is shown). Interestingly, upon LMα2 chain deficiency the truncated LMα1 chain was upregulated in all examined tissues (skeletal muscle, diaphragm, heart, peripheral nerve) compared to ΔE3 mice (Fig. 2A). Also, the expression levels seemed to reach those detected in dy3KLMα1 mice overexpressing full-length LMα1 chain. We also noted intracellular staining of truncated LMα1 chain in skeletal muscle (Fig. 2A). Western blot analyses with an antibody against LMα1LG3 domain revealed even higher expression (approximately 2.5-fold) of ΔE3LMα1 chain in dy3K/ΔE3 muscles compared to full-length LMα1 chain in dy3KLMα1 muscles (Fig. 2B). Therefore, we can rule out the possibility that the observed phenotype of dy3K/ΔE3 mice described below is due to insufficient expression of truncated LMα1 chain. Also, it is clear that the regulatory mechanisms involved in LMα1 chain transgene expression are complex. We also assessed the expression of LMα4 and α5 chains. We and others have previously shown that expression of these two LM chains is upregulated in LMα2 chain deficient basement membranes [25,39] (see also Figure S2). In dy3K/ΔE3 mice, the muscle basement membrane expression of LMα4 and α5 chains was very similar to that of dy3K/ΔK mice (Figure S2). Hence, we suggest that the compensatory increase of LMα4 and LMα5 chains has no beneficial effects in dy3K/ΔE3 muscles (which are analyzed in detail in the next paragraphs).

Expression of integrin α7B and dystroglycan in dy3K/ΔE3 tissues

We next evaluated the expression of integrin α7B and dystroglycan in dy3K/ΔE3 muscles. Expression of integrin α7B is reduced at the sarcolemma of dy3K/ΔK limb and heart muscle but to a lesser extent in dy3K/ΔK diaphragm (Fig. 3A). Notably, the expression of integrin α7B subunit was restored in dy3K/ΔE3 limb, diaphragm and heart muscle (Fig. 3A). Similarly, also full-length LMα1 chain reconstituted integrin α7B chain at LMα2 chain deficient sarcolemma [32]. We further detected an approximately 4.5-fold upregulation of integrin α7B in dy3K/ΔE3 skeletal muscle by immunoblotting experiments (Fig. 3B).

LMα2 chain deficiency does not significantly alter α-dystroglycan glycosylation and β-dystroglycan expression at the sarcolemma [32], probably because other ligands (e.g. perlecans) are still present. By immunofluorescence analyses, expression of α-dystroglycan also appeared normal in dy3K/ΔE3 limb, diaphragm and heart muscle and in sciatic nerve (Fig. 4A). In addition, we quantified expression of α- and β-dystroglycan and they remained the same in dy3K/ΔE3 vs. control skeletal muscle (Fig. 4B).

All in all, these results suggest that integrin α7B is increased, whereas dystroglycans appear normally expressed in dy3K/ΔE3 muscles.

Dy3K/dy3K mice with ΔE3LMα1 transgene have improved overall health

Dy3K/dy3K mice completely deficient in LMα2 chain were previously described [2]. Briefly, these animals suffer from severe muscle wasting, growth retardation, peripheral neuropathy and die approximately 3–5 weeks after birth. As shown in Fig. 5, the overall health of dy3K/ΔE3 mice was improved compared to dy3K/dy3K mice. First, dy3K/ΔE3 mice live longer. As demonstrated by the survival curve, approximately 75% of dy3K/ΔE3 animals survive up to 3 months (Fig. 5B). Further estimation of dy3K/ΔE3 survival encountered obstacles. Due to hindleg paralysis, several of them were sacrificed according to the guidelines of the ethical permit. Nevertheless, many dy3K/ΔE3 mice survive much longer than 3 months. Our oldest animals died one year after birth.

Second, dy3K/ΔE3 animals are bigger than dy3K/dy3K mice. At 2 weeks of age, dy3K/ΔE3 mice can be identified due to their growth retardation whereas dy3K/ΔE3 mice appeared outwardly normal (data not shown). Furthermore, the majority of dy3K/ΔE3 animals at 5 weeks of age can not be distinguished from normal littermates (Fig. 5A). Weight gain for female and male dy3K/ΔE3 mice was greatly delayed in 5-week-old mice whereas the weight gain for female and male dy3K/ΔE3 mice was significantly increased compared to dy3K/dy3K mice (Fig. 5C and data not shown). However, dy3K/ΔE3 mice weigh significantly less than normal littermates (Fig. 5C and data not shown). Beginning from 5 weeks of age, the difference in overall phenotype between most of dy3K/ΔE3 and wild-type mice became more evident. Many dy3K/ΔE3 animals are visibly smaller than control littermates (Fig. 5A, middle panel). However, some of the older dy3K/ΔE3 animals look outwardly normal and are almost indistinguishable from their littermates (Fig. 5A, left panel). Also, the ratio of quadriceps and tibialis anterior wet weight per body weight was similar in control and dy3K/ΔE3 mice, whereas the ratio was significantly reduced in dy3K/dy3K mice (Fig. 5D and data not shown). Hence, muscle mass was maintained in proportion to the body size in dy3K/ΔE3 mice. Nevertheless, most of dy3K/ΔE3 mice display severe peripheral nerve abnormalities, as demonstrated by temporary hindleg paralysis (either one or occasionally two limbs) (Fig. 5A, arrow). When lifted by the tail, they retract their hindlimbs toward the body. Still, dy3K/ΔE3 mice perform much better in the locomotion activity test compared to dy3K/dy3K animals (Fig. 5E), indicating that muscle function is largely improved.
preserved. Yet, $dy^{3K}/\delta E3$ mice move significantly less than control mice and this is supposedly due to the temporary paralysis (Fig. 5E). Finally, we noted that serum kinase activity was significantly elevated in $dy^{3K}/\delta E3$ mice (Fig. 5F), indicating that muscles may be dystrophic, despite improved general health.

In summary, survival during the first months of life and other features of the overall phenotype of $dy^{3K}/\delta E3$ mice are not greatly dependent on LM$\alpha 1LG4-5$.

$\Delta E3LM\alpha 1$ transgene reduces the dystrophic pathology of skeletal muscles and significantly prevents dystrophic changes in diaphragm and heart

We next examined the morphology of $dy^{3K}/\delta E3$ skeletal and heart muscle. When isolating skeletal muscles from $dy^{3K}/\delta E3$ mice (5-week-old and 4-month-old and older), it could be macroscopically seen that muscles were only modestly wasted (see also Fig. 5D). However, histological analyses of muscle revealed vast

Figure 3. Restoration and upregulation of integrin $\alpha 7B$ subunit in $dy^{3K}/\delta E3$ muscles. (A) Cross-sections of limb muscle (Li M), diaphragm (Dia) and heart from wild-type, $dy^{3K}/dy^{3K}$ and $dy^{3K}/\delta E3$ mice were stained with antibodies against integrin $\alpha 7B$. Bars, 50 $\mu$m. (B) Immunoblotting of total protein lysates from wild-type and $dy^{3K}/\delta E3$ skeletal muscle and quantitative measurement of integrin $\alpha 7B$ expression. There is approximately 4.5-fold more integrin $\alpha 7B$ in $dy^{3K}/\delta E3$ skeletal muscle ($p = 0.0231$). Results are shown as means $\pm$ SEM.

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Figure 4. Normal expression of dystroglycans in dy3K/dE3 muscles. (A) Cross-sections of limb muscle (Li M), diaphragm (Dia), heart and sciatic nerve (SN) from wild-type, dy3K/dy3K and dy3K/dE3 mice were stained with antibody IIH6 against α-dystroglycan. Bars, 50 μm. (B) Immunoblotting of glycoprotein preparations from wild-type and dy3K/dE3 skeletal muscle and quantitative measurement of α- and β-dystroglycan expression. Results are shown as means ± SEM. No significant difference in expression of α- and β-dystroglycan was noted between wild-type and dy3K/dE3 muscle (p = 0.8200 and p = 0.7527, respectively).

doi:10.1371/journal.pone.0011549.g004
regeneration of muscle fibers in limb muscles, demonstrated by the presence of small fibers with centrally located nuclei (Fig. 6A). Approximately 35% and 25% of 4–6-week-old dy3K/dyE3 quadriceps and triceps muscle fibers, respectively, contained centrally located nuclei and the numbers of centrally nucleated fibers did not differ significantly from dy3K/dy3K muscles (data not shown). The number of fibers in randomly selected areas was similar in wild-type and dy3K/dyE3 quadriceps muscle, but with a tendency of more fibers in dy3K/dyE3 mice (probably due to the presence of smaller regenerating fibers). Interestingly, a similar number of fibers was also noted in dy3K/dy1K quadriceps muscle (Figure S3). However, average fiber diameter is smaller (data not shown) and instead muscle contains fibrotic tissue (see Figure 8A). The number of fibers with centrally located was even higher in limb muscles of 4-month-old dy3K/dyE3 animals, indicating that pathology worsens over time (Fig. 6A and data not shown). Nevertheless, these results indicate that dy3K/dyE3 muscles undergo damage but that the constant regeneration and muscle mass is maintained with age. Moreover, the diaphragm did not undergo degeneration/regeneration cycles and its morphology appeared near normal in 5-week-old and 4-month-old animals (Fig. 6A-C). Dy3K/dy3K diaphragm at 4–6-weeks of age displayed about 16% of regenerated muscle fibers with central nuclei. A significant reduction was found in dy3K/dyE3 diaphragm, both in young and older animals and the numbers did not differ significantly from wild-type diaphragm (Fig. 6B). We also determined the muscle fiber size in 4–6-week-old diaphragm muscle. The fiber size distribution was shifted towards smaller fiber sizes in dy3K/dy3K animals, compared with wild-type muscles. Notably, the shift was largely prevented in dy3K/dyE3 muscles (Fig. 6C).

To demonstrate functional benefit conferred by the truncated LMz1 chain in diaphragm, we subjected dy3K/dyE3 mice to downhill treadmill exercise and sarcocellular integrity was evaluated by Evans blue dye (EBD) accumulation. It has previously been shown that only occasional EBD-positive fibers are found in dy/dy muscles [40]. In agreement with these results, we also detected a few EBD-positive fibers in unexercised dy3K/dy3K muscles. We also observed a few EBD-positive fibers in unexercised dy3K/dyE3 limb muscles, but almost none in dy3K/dyE3 diaphragm (Fig. 7A). While it was not possible to exercise dy3K/dy1K animals, dy3K/dyE3 limb muscles were susceptible to exercise-induced sarcocellular injury as evidenced by increased uptake of EBD. Interestingly, downhill running induced very little damage in dy3K/dyE3 diaphragm (Fig. 7A). Although EBD uptake in exercised dy3K/dyE3 limb muscles varied, both between animals and opposing limbs within the same animal, the diaphragm was consistently unaffected. Hence, truncated LMz1 chain prevents exercise-induced injury in diaphragm but not in limb muscles, indicating that different muscles have different requirements for LMz1LG4-5 domains.

The phenomenon of progressive muscle fiber damage in the limbs was further underscored by caspase-3 staining. Apoptosis has been shown to contribute to the severe dystrophic changes in muscles from MDC1A patients and LM22 chain deficient mice [2,41,42]. In both dy3K/dy3K and dy3K/dyE3 mice either single or multiple EBD-positive fibers were detected or apoptosis was more robust (Fig. 7B). In contrast, the muscles from LM22 chain deficient mice overexpressing full-length LM21 chain (dy3K/dyELM21) were free of apoptotic fibers (no caspase-3 staining was observed, Fig. 7B). Interestingly, apoptosis did not take place in dy3K/dyE3 diaphragms, whereas apoptotic fibers were present in diaphragms from dy3K/dyE3 mice (Fig. 7B). This data strongly suggests that LMz1LG4-5 protects limb muscles from apoptosis, most probably via dystroglycan binding, whereas truncated LMz1 chain is sufficient to prevent apoptosis in diaphragm muscle fibers.

Regardless of apoptotic cell death, muscle replacement with connective tissue, so evident in dy3K/dyE3 mice [25], was not very obvious in dy3K/dyE3 muscles (Fig. 6A). This tendency was also demonstrated by tenascin-C staining. Tenascin-C has been shown to be upregulated and extends to the interstitium between muscle fibers in dy/dy and dy3K/dy1K mice [25,43]. Some muscles from different dy3K/dyE3 animals showed moderate upregulation of tenascin-C (Fig. 8A, two individuals are shown, four animals were analyzed). However, tenascin-C expression was less pronounced than in dy3K/dy1K muscles. Also, some dy3K/dyE3 limb muscles did not display tenascin-C upregulation (Fig. 8A). Moreover, diaphragm did not show any signs of fibrosis (Fig. 8A).

Cardiomyopathy is not a major feature of MDC1A [1]. However, 2-month-old dy3K/dyE3 hearts show infiltration of connective tissue [44]. Dy3K/dy3K mice probably die too early in order to develop heart fibrosis (data not shown). Therefore, we compared 5–6-month-old dy3K/dyE3 hearts with hearts from 8-week-old dy/dy mice, which show massive fibrosis in the ventricle wall (Fig. 8B). As demonstrated by hematoxylin and eosin staining, dy3K/dyE3 hearts did not display any fibrotic lesions (Fig. 8). This trend was further confirmed by absence of tenascin-C staining (Fig. 8B).

In summary, LMz1LG4-5 domains are important for securing the mechanical stability of limb muscle fibers in LM22 chain deficiency, most probably by binding to dystroglycan. Interestingly, LMz1LG4-5 domains are not involved in improvement of diaphragm and heart muscle morphology, indicating that other sites of LMz1 chain (most likely integrin α7β1 binding modules) are responsible for functional replacement of LM22 chain in these muscles.

Skeletal muscle regeneration is not impaired in dy3K/dyE3 mice

Since muscle regeneration seemed to be continuously maintained in dy3K/dyE3 limb muscles (Fig. 6A), we next analyzed their regenerative properties in more detail. We injected 2–3-month-old control, dy3K/dyE3 mice and 3-week-old dy3K/dy3K tibialis anterior with cardiotoxin to induce muscle damage and trigger muscle
regeneration. Four days after injection many new fibers had reformed in all mice examined (data not shown). These fibers expressed embryonic myosin heavy chain, indicating an ongoing regeneration (Fig. 9B). Surprisingly, the regeneration process clearly took place in the absence of LMα2 chain (although newly formed muscle cells in dyK/dyK tibialis anterior were rather small) (Fig. 9B). Tibialis anterior from dyK/δE3 mice also showed normal initial regeneration, comparable to control mice. Most importantly, after 11 days post injection, dyK/δE3 muscles displayed the regeneration pattern characteristic for control mice.
Figure 7. EBD staining of unexercised and exercised muscles and caspase-3 immunostaining. (A) Three- to 5-week-old dy3K/dy3K mice (not exercised) display a few EBD-positive fibers. Also, unexercised dy3K/d3E3 limb muscles display few fibers positive for EBD, whereas hardly any are detected in dy3K/d3E3 diaphragm. Dy3K/dy3K mice were not in the condition to be exercised on the treadmill, but 7–13-week-old dy3K/d3E3 mice were analyzed for EBD uptake upon exercise. Increased uptake of EBD is seen in exercised dy3K/d3E3 limb muscles, but truncated LMα1 chain prevents exercise-induced injury in diaphragm. Bar, 200 μm. (B) Robust expression of caspase-3 (green) in the fibers from dy3K/dy3K and dy3K/d3E3 limb muscles

B

WT  dy3K/dy3K  dy3Kd3E3  dy3KLMα1

Li M

Dia
and they were not distinguishable from each other (Fig. 9A). Injected dy/−/−ΔE3 tibialis anterior muscles were tightly packed with big fibers. Also, the expression of embryonic myosin heavy chain was not detected after 11 days (Fig. 9B). This data confirms that regeneration in the presence of truncated LM1 chain is characterized with high capacity and maintenance. The regeneration in dy/−/−/ΔE3 mice was delayed and not as well-organized as in control and dy/−/−ΔE3 animals, since the muscle fibers in LM2 chain deficient mice appeared to be less packed and surrounded by connective tissue (Fig. 9A). Also, single fibers still expressed embryonic myosin heavy chain.

In summary, these data provide more insight into mechanism of muscle regeneration in LM2 chain deficiency and indicate that LM2 chain deprived of LG4-5 domains ensures proper regeneration. Therefore, binding to dystroglycan is not essential to ensure sufficient muscle regeneration and its maintenance.

LM2/1LG4-5 is essential for myelination in peripheral nervous system in LM2 chain deficiency

MDC1A patients as well as dy/−/−/ΔE3 mice display dysmyelination neuropathy that leads to reduced conduction velocity of nerve impulses [45–47]. Unmyelinated axon bundles are prominent especially in spinal roots of LM2 chain deficient mice. We have demonstrated before that overexpression of full-length LM1 chain in dy/−/−/ΔE3 peripheral nervous system largely corrects myelination defects [27]. Dy/−/−ΔE3 mice display hindleg paralysis and motor dysfunction. Morphology analyses of spinal roots and sciatic nerves confirmed that overexpression of truncated LM1 chain did not correct the phenotype of the proximal part of peripheral nervous system. In spite of the presence of truncated LM1 chain in both dorsal and ventral roots, large areas with unmyelinated axons (indicating incomplete axonal sorting) were evident in dy/−/−ΔE3 mice (Fig. 10). Similar bundles of naked, unmyelinated axons have also been described in dorsal and ventral roots of dy/−/−ΔE3 mice [27]. Importantly, this process was fully prevented upon overexpression of full-length LM1 chain in LM2/1 chain deficient peripheral nervous system [27], suggesting a role for LG4-5 domains in myelination processes.

Although myelination took place in the distal part of dy/−/−ΔE3 peripheral nervous system, sciatric nerve morphology was only partially rescued compared to dy/−/−/ΔE3 mice. Bundles of unsorted unmyelinated axons have been reported in dy/−/−/ΔE3 sciatric nerve [27] (see Fig. 10). Smaller, yet clearly visible patches of unsorted axons were also detected in dy/−/−ΔE3 sciatric nerves (Fig. 10 and 11). While occasional unmyelinated axons are present in normal animals (Fig. 11, top panel) and they are known to be part of a healthy nerve, the bundles present in dy/−/−ΔE3 nerves were clearly bigger (Fig. 11, top panel) and more frequent (data not shown), than in control mice. Tomacula (thickened myelin sheaths) was observed in dy/− mice [48] and we also detected these hypermyelinated axons in dy/−/−/ΔE3 animals (Fig. 10). Fewer tomacula were seen in dy/−ΔE3 mice (Fig. 10). Electron microscopy analyses of 2–4-month-old dy/−/−ΔE3 sciatric nerves revealed a whole spectrum of pathologies. Apart from axons with normal appearance (Fig. 11, top panel, yellow star), many axons with myelin distortion and/or abnormal ovoid shape were detected, especially in the animals affected more severely with
Figure 8. Analyses of fibrosis in skeletal muscle and heart. (A) Different wild-type (4-month-old), dy3K/dy3K (4-week-old) and dy3K/dE3 (4-month-old) muscles (gastrocnemius, triceps, diaphragm) were stained with an antibody against tenascin-C. Occasionally tenascin-C is present in interstitial matrix of limb dy3K/dE3 muscles, but it is absent from diaphragm. Note extensive tissue fibrosis in dy3K/dE3 muscles. Four dy3K/dE3 animals were analyzed. Bars, 50 μm. (B) Hematoxylin and eosin staining (upper panel) of hearts from wild-type (5–6-month-old), dy/dy (8-week-old) and dy3K/dE3 (5–6-month-old) mice. Hearts from dy/dy mice displayed localized or extensive fibrosis in the ventricular wall. Dy3K/dE3 hearts did not exhibit any defects and looked as wild-type controls. Tenascin-C immunolabelling confirms the presence of fibrotic lesions in dy/dy hearts and their absence in dy3K/dE3 hearts (lower panel). Three animals from each group were analyzed. Bars, 50 μm.

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In summary, these data show that LMα1LG4-5 is partially required for basement membrane assembly and cell surface anchoring in the neuromuscular system.

Discussion

In this paper, we investigated the roles of LM C-terminal globular domains (and accordingly LM receptors dystroglycan and integrin α7B) in muscle and nerve and analyzed the molecular mechanisms underlying LMα1 chain mediated rescue of LMα2 chain deficiency.

LMα1LG4-5 is dispensable for improvement of diaphragm and heart muscles, but not limb muscles in LMα1 chain rescued mice

Overexpression of LMα1 chain lacking LG4-5 domains in dy3K/dy3K mice resulted in significantly prolonged lifespan (at least tripled compared with dy3K/dy3K mice). Cardiopulmonary complications are often responsible for the early death in dystrophic patients but cardiomyopathy is not a common feature of LMα2 chain deficiency [1]. Considering that a severely dystrophic diaphragm will lead to pulmonary dysfunction, it is quite likely that the improved diaphragm in dy3K/dy3K mice accounts for the increased survival, although we can not completely exclude that the expression of truncated LMα1 in other tissues (e.g. heart) is beneficial. Importantly, integrin α7B subunit is absent from dy3K/dy3K sarcolemma, but reconstituted in dy3K/δE3 muscles. Hence, we propose that prolonged lifespan is secured via LMα1LG1-3 binding, most probably to integrin α7β1, in the diaphragm and possibly in the heart.

Interestingly, while LMα1LG4-5 turned out to be dispensable for diaphragm and heart muscle, overexpression of LMα1 chain devoid of LG4-5 did not secure the complete correction of LMα2 chain deficient limb muscles. Although it is not surprising that LMα2 chain deficient peripheral nerve and muscle could respond differently to δE3LMα1 overexpression, it is somewhat unexpected that limb muscles and diaphragm would not be spared to the same degree, indicating an important difference in their properties or molecular signature in response to lack of a single protein domain. Our results also point toward diverse roles of LMα1LG4-5 when expressed in different muscle groups. For example, apoptosis has been shown to contribute to LMα2 chain deficient pathogenesis [54,55]. In limb skeletal muscle, LMα1LG4-5 appeared to be critical for prevention of apoptosis of muscle fibers. However, this was not the case in diaphragm. Integrin α7β1 has been considered to be the major mediator of myofiber survival [29]. Now, we suggest that also LM binding to dystroglycan prevents apoptosis in limb...
muscle fibers. In support of this notion, dystroglycan binding to LMα2 chain has been shown to protect muscle cells in culture from apoptosis [56]. Yet, in some muscles, (e.g. diaphragm) integrin α7β1 could be the key player in apoptosis prevention.

LMα1LG4-5 is not involved in muscle regeneration in LMα1 chain rescued mice

Skeletal muscle regeneration depends on satellite cells, which express both dystroglycan and integrin α7β1 [10,57]. In spite of muscle damage and cell death, dy3K/δE3 muscles were able to regenerate and maintain muscle mass, both in normal conditions and when subjected to cardiotoxin injection. Also, mini-agrin increases the regenerative capacity of LMα2 chain deficient muscles. Since mini-agrin binds dystroglycan (rather than integrin α7β1), it is hypothesized that mini-agrin binding to dystroglycan is responsible for the restored regeneration [58,59] and it has been demonstrated that dystroglycan activity in satellite cells is crucial for the maintenance of regeneration [10]. Yet, integrin α7 chain is also involved in skeletal muscle regeneration, as α7 integrin-null mice subjected to cardiotoxin injections exhibit a profound delay in muscle regeneration [57]. Hence, integrin α7 chain is most likely responsible for efficient muscle regeneration in dy3K/δE3

Figure 10. Analyses of myelination in peripheral nervous system. (A) Toluidine blue staining of ventral and dorsal roots and sciatic nerves from 2–4-month-old normal and dy3K/δE3 mice and 5-week-old dy3K/δK animals. Myelination defects are clearly visible in dy3K/δE3 and dy3K/δK spinal roots with distinct and wide-spread unmyelinated axons bundles. Occasional unmyelinated axon bundles are also detected in sciatic nerve of dy3K/δE3 and dy3K/δK mice (indicated with arrows). Arrowheads denote tomacula. (B) Truncated LMα1 chain is present in dy3K/δE3 spinal roots as demonstrated by immunostaining using the antibody against N-terminal (green) and LG4 domain no staining). Four animals from each group were analyzed. Bars, 25 μm.
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mice since the dystroglycan binding domain is missing. We propose that the most aggravating step in MDC1A might be the lack of efficient regeneration due to abolished LMα2-integrin α7 interaction rather than impaired LMα2-dystroglycan interaction.

LMα1LG4-5 is vital for myelination in peripheral nerve in LMα1 chain rescued mice

None of the neuronal symptoms that occur in LMα2 chain deficiency were ameliorated by δE3LMα1 overexpression. This data together with our previous work [27] indicates a very important role for LMα1LG4-5 in LMα1 chain rescued peripheral nervous system. Interestingly, the phenotype of dy3K/dyE3 and dy3K/E3 peripheral nervous system does not resemble the phenotype of any conditional knockout mice, where major LM receptors (dystroglycan, integrins β1 and β4) were depleted from Schwann cells [18–20,60]. Furthermore, genetic inactivation of the α7 integrin chain does not affect peripheral nerve morphology and function [60]. Therefore, those receptors might just regulate the LMα2 chain/LMα1 chain interaction together with other receptors. Heparan sulfate proteoglycans syndecans presumably bind LMα1 via the LG4 domain [61] and are enriched in Schwann cells [62], but syndecan-null mice do not display peripheral nerve defects [63]. Also, sulfatides have been shown to bind LMα1LG4-5 [64] and LMα2LG4-5 [65,66] and to be expressed in peripheral nerves [67], where they mediate basement membrane assembly and dystroglycan and integrin signaling [68]. Strikingly, lack of sulfatides

Figure 11. Detailed analyses of morphology and properties of 2–4-month-old dy3K/E3 sciatic nerves (electron microscopy). Top panel: Unsorted axons in wild-type (WT) and dy3K/E3 sciatic nerves. Most of bundles of unmyelinated axons are bigger in dy3K/E3 mice (enlarged panels). Apart from unsorted axons (red arrow, overview panel), many compressed, ovoid axons, often with convoluted outfoldings and redundant loops are seen (green arrowhead). Yet, numerous normally shaped and myelinated axons are present (yellow star). Single macrophages were detected (blue arrow). Middle panel: Myelin defects linked to axonal degeneration. (A) Overview of a pathological area with different stages of myelin abnormalities, myelin degradation and axonal degeneration. Red arrowhead - degenerating axon. Blue arrow - degraded interaxonal myelin leading to axon degeneration. Green arrow - axons with vesicular or lamellar myelin debris (intrusions) and dense bodies, often being signs of early stage of degeneration. (B–E) Detailed photos of different forms of degenerating axons found in various areas of sciatic nerve. (B) Degenerating axon with interaxonal myelin debris. (C) An almost completely demyelinated nerve fiber is filled with dilated smooth endoplasmic reticulum and degenerated mitochondria and undergoes degeneration. (D) Granular myelin degeneration with numerous myelin breaks. Arrowhead indicates myelin outfoldings/redundant loop formation. (E) Axonal degeneration forgoes myelin degradation as indicated by loose non-degraded myelin swirls. Schwann cell detached from empty myelin is indicated with arrow. Bottom panels: various axonal and myelin distortions rooting from incorrect myelination process and/or disruption of Schwann cell properties after myelination. (F) One Schwann cell (S) contains thinly myelinated axon (a) with vacuole (v), swollen myelin debris (arrowhead) and thickened myelin sheaths of minute axons (arrow) or myelin outfoldings. (G) Satellite myelinated axon within a bigger axon or excessive intramyelin fold. Myelin outfoldings and satellite myelination seen in F and G may result from impaired myelination process. (H) Redundant loop formation. (I) Hypermyelination due to excessive redundant loop formation. (J) Tomacula. (K) Onion bulb. Arrow indicates an almost demyelinated axon. Bar, 3 µm.

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and galactocerebrosides (another type of glycolipids) in mice results in similar myelin abnormalities in central nervous system as in dy3K/dy3K distal peripheral nervous system. Hence, the LM receptor might belong to glycolipids [69–71]. Furthermore, monosialoganglioside GM1 has been shown to bind LM-111 and promote neurite outgrowth [72]. Therefore, the identification of a peripheral nerve LM receptor is an exciting task.

Basement membrane assembly in LMα1 chain rescued mice requires LMα1LG4-5

In early studies of LMα2 chain deficiency, lack of basement membranes was considered to be deleterious to the muscle fibers [2,52,73,74] and to represent one of the MDC1A pathogenic mechanisms. Consequently, the approach of basement membrane restoration has been hypothesized to be beneficial for the

**Figure 12. Basement membranes in the neuromuscular system in the absence of LMα1LG4-5.** Electron microscopy of (A) limb skeletal muscle (wild-type and dy3K/dE3); (B) diaphragm (wild-type, dy3K/dy3K and dy3K/dE3); (C) heart (wild-type, dy3K/dy3K and dy3K/dE3); (D) sciatic nerve (wild-type and dy3K/dE3). In dy3K/dE3 limb skeletal muscle basement membranes had patchy appearance as compared to continuous basement membranes in wild-type samples (A) (arrowheads, in all figures). Stars depict the areas with lack of basement membrane in all figures. In dy3K/dy3K diaphragm basement membranes are either patchy or completely absent. Presence of truncated LMα1 chain partially restores basement membranes in the diaphragm (B). Similarly, in LMα2 chain deficient heart basement membranes are disrupted and partially restored upon dE3LMα1 chain overexpression (C). Basement membranes were locally patchy around dy3K/dE3 Schwann cells (Sch), but also sometimes continuous throughout longer distances (D). Four animals from each group were analyzed. Bars, 400 nm.

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improvement of the dystrophic muscle phenotype [25,28,44,53]. Yet, continuous basement membranes are not strictly required for myelination in peripheral nervous system [46,75]. Likewise, basement membranes are also patchy or less dense in dystrophic LM α1-δE3 mice diaphragm and heart muscle, indicating that continuous basement membranes are not vital for the complete correction of the dystrophic phenotype.

Our data helps to further understand the involvement of LM α1LG4-5 and LG1-3 in basement membrane assembly and point toward interesting basement membrane scaffolding mechanisms in the neuromuscular system in the absence of LM α1LG4-5. Exogenous LM α1LG4-5 has been shown to totally abolish the formation of basement membranes in vitro where it selectively blocked the cell-surface accumulation of a LM network [68,76,77]. In our in vivo model, despite lack of LM α1LG4-5, basement membranes showed only partial defects in cell surface anchoring. It is not excluded that integrins or other receptors that bind LM α1LG4-1-3, partially could compensate for lack of LM α1LG4-5 domain and dystroglycan/sulfatide binding and anchor the LM network to the cell surface. This accumulation, however, is not as efficient as in the presence of full-length LM α1 chain or mini-agrin [25,27,44,53], as basement membranes appear to be continuous only locally in dystrophic LM α1 mice. Therefore, it is possible that all LM α1LG domains and the cooperation between different LM α1LG1-5 receptors are important for the assembly of continuous basement membranes in vivo. This hypothesis is further substantiated in McKee et al., where all LG domains were shown to support LM tethering to cell surface [78,79]. However, very recent data by Han et al. [15] confirms that dystroglycan, but not integrin α7β1, is involved in basement membrane anchorage and maintenance (rather than actual assembly) in muscle. Therefore, LM α1LG4-5 binding to dystroglycan could be important not only for basement membrane assembly in the muscle, but also for its maintenance.

**Supporting Information**

**Figure S1** Expression of δE3LM α1 chain in limb skeletal muscle (SM), peripheral nerve (SN) and heart (He) of δE3 transgenic mice from lines No. 3 and 4. The two antibodies to detect truncated LM α1 chain were mAb200 and 1057+, which bind LG4 and N-terminal domains, respectively. Mosaic expression of δE3LM α1 chain was detected in transgenic neuromuscular tissues. Wild-type (WT) mice and full-length LM α1 chain transgenic animals (LMα1TG) were used as controls. Bars, 50 μm.

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**Figure S2** Immunostaining of LM α4 and α5 chains. Cross-sections of quadriceps femoris (Quad), triceps brachii (Tri) and diaphragm (Dia) from 6-week-old wild-type, dy3K/dy3K and dy3K/δE3 mice were stained with antibodies against LM α4 chain (A) and α5 chain (B), respectively. Expression of LM α4 and α5 chains is increased at the muscle basement area in dy3K/dy3K mice and remained increased in dy3K/δE3 muscles. Four dy3K/δE3 animals were analyzed. Bar, 50 μm.

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**Figure S3** The numbers of fibers in a randomly selected area is not significantly different between the genotypes.

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**Author Contributions**

Conceived and designed the experiments: KIG MA VC HE MD. Performed the experiments: KIG MA VC HE MD. Analyzed the data: KIG MD. Wrote the paper: KIG MD.

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