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_J Clin Invest._ 2017;127(10):3730-3740. [https://doi.org/10.1172/JCI93445](https://doi.org/10.1172/JCI93445).

Maintenance of muscle structure and function depends on the precise organization of contractile proteins into sarcomeres and coupling of the contractile apparatus to the sarcoplasmic reticulum (SR), which serves as the reservoir for calcium required for contraction. Several members of the Kelch superfamily of proteins, which modulate protein stability as substrate-specific adaptors for ubiquitination, have been implicated in sarcomere formation. The Kelch protein Klhl31 is expressed in a muscle-specific manner under control of the transcription factor MEF2. To explore its functions in vivo, we created a mouse model of Klhl31 loss of function using the CRISPR-Cas9 system. Mice lacking Klhl31 exhibited stunted postnatal skeletal muscle growth, centronuclear myopathy, central cores, Z-disc streaming, and SR dilation. We used proteomics to identify several candidate Klhl31 substrates, including Filamin-C (FlnC). In the _Klhl31_-knockout mice, FlnC protein levels were highly upregulated with no change in transcription, and we further demonstrated that Klhl31 targets FlnC for ubiquitination and degradation. These findings highlight a role for Klhl31 in the maintenance of skeletal muscle structure and provide insight into the mechanisms underlying congenital myopathies.

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Deficiency in Kelch protein Klhl31 causes congenital myopathy in mice

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Introduction

Skeletal muscle development and postnatal growth depend on transcriptional activation of a broad array of muscle structural genes and maintenance of protein homeostasis in an environment that is subject to intensive wear and tear. The establishment and integrity of the sarcomere, the smallest functional contractile unit of striated muscle, are vital for muscle function. Mutations in genes that encode sarcomere proteins often result in lethal or debilitating myopathies, including muscular dystrophies and nondystrophic congenital myopathies (1–3). Congenital myopathies typically manifest around birth and are characterized by structural abnormalities, including nemaline rods, centralized nuclei, and central cores (4). To date, there are no effective therapies for any of these disorders, and much remains to be learned about the underlying disease mechanisms.

Mutations in the Kelch-like proteins KBTBD13, KLHL40, and KLHL41 result in nemaline myopathy (5–8). Several mutations in a gene that was significantly downregulated in differentiated myoblasts isolated from mice with satellite cell–specific deletion of Mef2a, Mef2c, and Mef2d (13). The mouse Klhl31 promoter contains 3 MEF2 consensus-binding sites, one of which is conserved to human at approximately 500 bp upstream of the transcriptional start site (Supplemental Figure 1A; supplemental material available... 

Results

Skeletal muscle–specific expression of Klhl31. We discovered Klhl31 as a gene that was significantly downregulated in differentiated myoblasts isolated from mice with satellite cell–specific deletion of Mef2a, Mef2c, and Mef2d (13). Klhl31 localizes to the sarcomere Z-disc, and by using 2 unbiased approaches, we identified several potential Klhl31 substrates, including Filamin-C (FlnC). These findings highlight a role for Klhl31 in maintenance of skeletal muscle structure and provide insight into the mechanisms underlying congenital myopathies.
Klhl31 KO mice were born seemingly healthy, but they showed a reduction in body weight beginning around P10 (Figure 2D), and between P14 and P21, the KO mice were visibly distinguishable from their WT littermates (Figure 2E). The change in body weight coincided with a decrease in the mean cross-sectional area (CSA) of myofibers at P10, indicative of impaired postnatal muscle growth (Figure 3, A and B). To determine whether the loss of body weight was due to a decrease in muscle weight or the weight of other organs, we weighed several different muscle groups and organs in 6-week-old mice. Every muscle group tested weighed significantly less in the KO mice compared with WT, while other organ weights were unchanged (Supplemental Figure 2, A–D). Although the KO mice demonstrated hypotrophic myofibers by P10, further histological analysis revealed normal myonuclear positioning and unaltered H&E and desmin staining (Supplemental Figure 3, A and B). However, by 4 weeks of age, in addition to having hypotrophic myofibers, KO mice began to exhibit an accumulation of internalized myonuclei and pathological desmin aggregation (Supplemental Figure 3C), which was further exacerbated by 12 weeks of age (Figure 3, C and D). Additionally, protein aggregates, centralized nuclei, and a paucity of sarcomeric proteins could easily be detected in longitudinal sections by H&E staining of quadriceps (Supplemental Figure 3D). Membrane proteins involved in muscular dystrophies (15, 16), however, were unperturbed (Supplemental Figure 4).

Assessment of the mitochondrial architecture and the sarcoplasmic reticulum (SR) networks by NADH-tetrazolium reductase (NADH-TR) staining revealed an increase in the number of oxidative fibers, core-like lesions, and rubbed out fibers in the KO mice.
by 4 weeks of age (Figure 3E). Electron microscopy also revealed an increase in the number of mitochondria in KO mice compared with WT littermate controls (Figure 3F), consistent with the increased NADH-TR staining. In addition, the KO mice contained clusters of myofibrils that lacked discernible myofilaments (Figure 3F), a feature that was even more pronounced in older animals (Figure 3G). Pleomorphic dense structures and Z-disc streaming were also present in the KO animals, and in some instances, the Z-disc was completely degenerated (Figure 3F). Older animals exhibited sarcomeric disarray, myofibril degeneration, and an immensely dilated SR network (Figure 3G and Supplemental Figure 5, A and B).

Next, we tested the biological effects of Klhl31 loss of function by subjecting 6-week-old male mice to grip-strength tests. Klhl31-KO mice showed a significant decrease in muscle strength in both the forelimbs and hind limbs compared with WT mice (Figure 3H). These findings indicate an essential role for Klhl31 in maintaining postnatal skeletal muscle integrity and function.

Klhl31 localizes predominantly to the Z-disc. Immunostaining of isolated myofibers showed colocalization of Klhl31 with desmin (Figure 4A) and a complete overlap with the Z-disc protein α-actinin (Figure 4B), suggesting that Klhl31 is localized to the Z-disc and cytoskeletal network. No immunostaining for Klhl31 was observed in isolated myofibers from Klhl31-KO mice (Supplemental Figure 6), confirming the specificity of the antibody. While we saw no nuclear localization of Klhl31 in vivo, a previous report suggested that Klhl31 localizes to the nucleus and cytoplasm when overexpressed in COS-7 cells (17). To test this and to further validate our antibody, we also transfected COS-7 cells with a myc-tagged Klhl31 expression plasmid and costained the cells using antibodies that recognize myc or Klhl31. When these cells were visualized, only the cells that were transfected with myc-Klhl31, as evidenced by myc staining, were immunoreactive to the Klhl31 antibody, highlighting the cytoplasmic localization and the specificity of the Klhl31 antibody (Figure 4C). As a control, and to be confident that our Klhl31 antibody did not detect myc, we transfected a myc-tagged Bag3 expression plasmid into COS-7 cells and similarly costained the cells with antibodies that recognize myc or Klhl31. Cells that were transfected with myc-Bag3 were positive for the myc signal, but were negative for any signal when stained with the Klhl31 antibody (Figure 4D). These findings demonstrate that the cytoplasmic Klhl31 protein predominantly localizes to the Z-disc in skeletal muscle. This localization differs from that of Klhl40, another muscle-specific kelch-like protein, which was reported to localize to the A- and I-bands (6–8).

Figure 2. CRISPR-Cas9 gene editing eliminates Klhl31 protein and causes body weight reduction in Klhl31-KO mice. (A) qRT-PCR of 6-week-old WT and Klhl31-KO quadriceps shows a significant reduction in the Klhl31 transcript (n = 3 for both genotypes). ***P < 0.001. (B) Western blot analysis showing loss of Klhl31 protein in skeletal muscle at P10. (C) Immunofluorescent detection of Klhl31 protein in P10 quadriceps of WT and Klhl31-KO mice. Scale bar: 20 μm. (D) Body weight curve of WT and Klhl31-KO mice (WT, n = 5; HET, n = 17; and KO, n = 5). *P < 0.05; **P < 0.01; ***P < 0.001, WT versus KO. Data are presented as mean ± SEM. (E) Representative images of 2-week-old Klhl31-KO mice with WT littermates. Statistical analyses were performed using an unpaired 2-tailed Student’s t test.
Figure 3. Loss of Klhl31 causes multiple muscle abnormalities. (A) Laminin immunostaining of P10 WT and Klhl31-KO quadriceps. Scale bar: 50 μm. (B) Quantification of the mean CSA of WT and KO (n = 4 for both genotypes). **P < 0.01. Data are represented as mean ± SEM. (C) H&E staining of quadriceps muscle from 12-week-old WT (left) and KO (right) mice. Scale bar: 50 μm. (D) Desmin immunostaining of quadriceps from 12-week-old WT (left) and KO (right) mice. Scale bar: 50 μm. (E) NADH-TR staining of quadriceps from 4-week-old animals reveals a disorganized intermyofibrillary matrix, central cores (white arrowheads), and rubbed out fibers (red arrowheads) in KO. Scale bar: 50 μm. (F and G) Transmission electron microscopic images of (F) 4-week-old animals. In transverse orientation (top panel), KO mice show myofilament loss (white arrowhead) and pleomorphic dense structures (arrows). Red arrowhead denotes a myofibril with a normal pattern of myofilaments. In longitudinal sections (lower panel), numerous mitochondria are evident, Z-line streaming (white arrowhead) is abundant, and numerous Z-discs are degenerated (red arrowheads). Scale bars: 0.5 μm (upper panel); 2 μm (lower panel). (G) Transverse sectioning through quadriceps of 7-month-old KO animals (top panel) reveals a severely dilated SR (white arrowhead). A profound loss of myofilaments is evident in KO mice. Longitudinal sectioning demonstrates a loss of the sarcomere architecture and Z-disc continuity (white arrowhead in bottom panel). Scale bars: 0.2 μm (upper panel); 0.5 μm (lower panel) (n = 3 for both genotypes). (H) Grip-force strength test of 6-week-old WT and KO mice. Each symbol represents the average grip force from 6 grip-force trials from an individual mouse. (n = 6 for both genotypes). **P < 0.01, ****P < 0.0001. Data are presented as mean ± SEM. Statistical analyses were performed using an unpaired 2-tailed Student’s t test.
Supplemental Methods). All but one of the differentially expressed proteins identified in the KO mice by proteomics were upregulated (Supplemental Table 1), suggesting that the main function of Klhl31 might be to target proteins for degradation. Six proteins in common were identified in the in vivo proteomics analysis and the TAP, including sarcomeric, SR, and mitochondrial proteins (Table 1). The differential expression of most of these proteins was confirmed by Western blot analysis and immunofluorescence (Figure 5B and Supplemental Figure 7). Moreover, the transcript levels of the identified proteins were unchanged or decreased, suggesting that the increase in protein levels was regulated posttranscriptionally (Figure 5C).

Sarcolemmal membrane-associated protein (Slmap) was the most upregulated protein identified by proteomics from the Klhl31-KO quadriceps (Supplemental Table 1), but was not identified by TAP in C2C12 myotubes. Slmap is a muscle-enriched, integral membrane protein and a component of the striatin-interacting phosphatase and kinase (STRIPAK) complex, which has been described as possessing roles in multiple cellular processes, including signaling, trafficking, cell migration, and cardiac function (18). Previous reports have shown that Slmap is involved in microtubule organization (19), myoblast fusion (20), and excitation-contraction coupling (21). It has also been reported that mutations in SLMAP may give rise to Brugada syndrome (22). Intriguingly, transgenic mice overexpressing Slmap specifically in cardiomyocytes display cardiac dysfunction and a dilated SR (23), similar to the abnormalities observed in the skeletal muscle of Klhl31-KO mice.

Flnc, upregulated during skeletal muscle growth 5 (Usmg5), and cytoskeletal associated protein 4 (Ckap4) were all found to be increased in abundance in Klhl31-KO muscle and identified by TAP. Flnc is a well-characterized Z-disc protein that repeatedly folds and unfolds during contraction, a process that makes Flnc prone to damage and require regular turnover (24). Usmg5, also
known as diabetes-associated protein in insulin-sensitive tissues (DAPIT), is a small, muscle-enriched 58- amino acid peptide that is part of the mitochondrial ATP synthase (25–27). In cell lines, Usmg5 overexpression causes mitochondrial dysfunction and glucose mishandling (28) and its overexpression is also seen in porcine models of Duchenne muscular dystrophy (29). Ckap4, also known as Climp-63, is a ubiquitously expressed protein and many functions have been attributed to it (30). Interestingly, the overexpression of Ckap4 has been consistently shown to cause dilation and rearrangement of the endoplasmic reticulum (31, 32), similar to the expanded and dilated SR in Klhl31-KO mice.

**Klhl31 acts as a CRL substrate adapter.** Since Klhl31 interacts with Cul3 and nearly all of the differentially expressed proteins in the KO mice were upregulated, it seems likely that Klhl31 functions primarily as a CRL substrate adapter by targeting proteins for degradation. To determine whether Klhl31 could interact with any of the proteins identified by TAP and proteomics, we performed coimmunoprecipitation experiments in transfected 293T cells. We were able to detect an interaction between Klhl31 and FlnC (Figure 6A); however, we were unable to demonstrate interaction between Klhl31 and Usmg5 or Slmap (Supplemental Figure 9, A–D). We therefore looked at the expression of FlnC and Slmap-5 proteins in younger animals and confirmed that FlnC upregulation precedes the increased levels of Slmap and Usmg5 in Klhl31-KO mice (Figure 6D).

**Discussion**

Skeletal muscle function requires the precise coordination of numerous proteins organized into the highly structured sarcomere. Consequently, mutations in a large number of sarcomeric proteins result in debilitating or lethal myopathies. Here, we identified the skeletal muscle–specific Kelch-like protein Klhl31 as a Z-disc protein and governor of postnatal skeletal muscle integrity. Disruption of Klhl31 in mice resulted in congenital myopathy with a heterogeneous collection of myopathic abnormalities, including stunted postnatal skeletal muscle growth, centronuclear myopathy, central cores, and rearrangement of the endoplasmic reticulum (31, 32), similar to the expanded and dilated SR in Klhl31-KO mice.

The upregulation of Slmap and Usmg5 is secondary. Furthermore, transgenic mice expressing Slmap (Slmap-TG) under the control of the skeletal muscle actin (SKA) promoter did not exhibit centronuclear myopathy or central core disease; however, Slmap-TG mice had significantly smaller myofibers (Supplemental Figure 9, A–D). We therefore looked at the expression of FlnC and Slmap-5 proteins in younger animals and confirmed that FlnC upregulation precedes the increased levels of Slmap and Usmg5 in Klhl31-KO mice (Figure 6D).

FlnC crosslinks actin at the Z-disc and interacts with multiple other proteins (36–40). The upregulation of FlnC has been observed in myofibrillar myopathy patients harboring disease-causing mutations in FlnC that cause misfolding and aggregation of the protein as well as the aggregation of several other proteins including Bag3 and heat-shock proteins (41), which were also iden-
identified by Klhl31 proteomics analyses. Chaperone-assisted selective autophagy (CASA) has been shown to control the removal of damaged Z-disc–associated proteins, including FlnC in skeletal muscle (24), while the cardiac-specific E3-ligase component F-box and leucine-rich repeat protein 22 (Fbxl22) has been demonstrated to control the Cullin-1–mediated turnover of FlnC in the heart (42). Given the abundant number of proteins associated with the Z-disc and the emerging role of this nexus as a signaling hub (43), it is not surprising that Z-disc–associated proteins would have different levels of proteostasis regulation.

Many of the upregulated proteins identified by proteomics in the Klhl31-KO mice are known to carry mutations that give rise to muscular dystrophies or congenital myopathies in humans, such as FlnC, αβ-crystallin, Bag3 (44), Dnajb6 (45), Lamin A/C (46), Dag1 (47), Sgcb (48), and Klhl41 (7). We cannot rule out that Klhl31 interacts with and facilitates the degradation of some of these proteins, which could account for some of the abnormalities seen in the Klhl31-KO mice, such as centronuclear myopathy. Many of these proteins also associate with the Z-disc, and further biochemical experiments are needed to determine whether Klhl31 promotes their degradation.

It is interesting that only 1 protein was identified as being downregulated in Klhl31-KO mice. A profound decrease in MybpC2 was detected and could either be attributed to (a) Klhl31 stabilizing MybpC2 in a manner similar to the effect of Klhl40 on LMOD3 and NEB (8), or, more likely, (b) the fact that following Z-disc instability in the KO mice, the sarcomere is compromised, leading to a secondary loss of thick filament proteins.

We examined the expression of proteins involved in centronuclear myopathies, including myotubularin, dynamin 2, and

Figure 6. Klhl31 targets FlnC for ubiquitination. (A) Coimmunoprecipitation assay showing Klhl31 interacts with FlnC. (B) Transfected COS-7 cells were immunoprecipitated with anti-Flag antibody, followed by Western blotting with anti-HA antibodies. Polyubiquitination of FlnC is reduced in the presence of a K48R mutant Ub or in the absence of Klhl31. FlnC protein levels were reduced by overexpression of Klhl31, Cul3, and Ub. This reduction is rescued by the addition of the K48R mutant Ub or in the absence of Klhl31. Black arrowhead indicates increased ubiquitinated FlnC with a reduction in polyubiquitination in the presence of the K48R mutant Ub. White arrowhead indicates a nonspecific band. (C) Immunohistochemistry of WT and Klhl31-KO quadriceps with α-actinin and FlnC antibodies shows FlnC aggregates in Klhl31-KO mice. Scale bar: 20 μm. (D) Western blot analysis of WT and Klhl31-KO quadriceps at P10 using FlnC, Slmap, and Usmg5 antibodies.
amphiphysin 2, but were unable to detect any changes in protein levels in the Klhl31-KO mice. However, proteomics did detect significant changes in nuclear membrane proteins, microtubule-associated proteins, and Klif5b (Supplemental Table 1), all of which have been implicated in myonuclear positioning (49–51). Future studies will determine whether Klhl31 plays a direct role in the microtubule dynamics required for normal positioning of myonuclei.

Mutations in Trim32, another sarcomeric E3 Ub ligase, also give rise to myofibrillar degeneration and SR dilation (52, 53), although both humans and mice with Trim32 loss of function have a much more profound sarcotubular myopathy, distinguished by large intermyofibrillar vacuoles. Like Klhl31, Trim32 has also been shown to localize to and target Z-disc proteins (52, 54), and we have identified potential downstream regulators that may cause the dilated SR observed in the Klhl31-KO mice. Both Ckap4 and Slmap were upregulated in the KO mice, which have both been shown to cause dilation and rearrangements of the ER (21, 31, 32).

In summary, we describe the functional characterization of a new protein involved in congenital myopathy. Klhl31 is a Z-disc protein that recruits FlnC for UPS-dependent degradation. The loss of Klhl31 in mice results in nondystrophic congenital myopathy, with hypotrophic fibers, Z-disc streaming, central cores, large intermyofibrillar vacuoles. Like Klhl31, Trim32 has also been shown to localize to and target Z-disc proteins (52, 54), and we have identified potential downstream regulators that may cause the dilated SR observed in the Klhl31-KO mice. Both Ckap4 and Slmap were upregulated in the KO mice, which have both been shown to cause dilation and rearrangements of the ER (21, 31, 32).

Methods

Generation of Klhl31-KO mice. KO mice were generated using the CRISPR-Cas9 gene-editing system by pronuclear and cytoplasmic injection of mouse embryos with Klhl31 gRNA and Cas9 mRNA as previously described (57). In short, a gRNA targeting the first exon of Klhl31 was cloned into PX330 (a gift from Feng Zhang, Massachusetts Institute of Technology, Boston, MA, USA; Addgene plasmid 42230) (58) and cleavage efficiency was tested in 10T1/2 mouse fibroblast cells. The gRNA was transcribed in vitro and spin-column purified, while the Cas9 mRNA was obtained commercially (TriLink Biotechnologies). Mouse embryos were injected with an equal ratio of gRNA and Cas9 mRNA into the pronucleus and cytoplasm and transferred to a surrogate dam for gestation. Mutants in the F1 generation were identified by TTE1 assay, and the alleles were cloned and sequenced. A mouse with a 2-bp insertion was chosen for further study and was backcrossed at least 4 generations on a C57BL/6 background. While both male and female mice exhibited similar myopathies, data from only male mice were reported. Mice were genotyped using a custom TaqMan genotyping assay (Life Technologies). Tail DNA was isolated on a heat block for 30 minutes using 100 μl of an alkaline lysis buffer (25 mM NaOH, 0.2 mM EDTA), followed by 100 μl of neutralization buffer (40 mM Tris-HCl). Particulates were removed by high-speed centrifugation, and the supernatant was diluted 1:10 in nuclease-free water. The DNA was then analyzed by qPCR with a mixture of the following oligonucleotides: F, CTTTAGGCGTACCCGACAGT; R, AGCAGAATGTAGCTTCTCTATGGT; WT probe, TTTCCTGTTAAGCAT-FAM; KO probe, TTTCCTGTTAAGCAT-VIC.

Generation of SKA-Slmap transgenic mice. A SKA-Slmap transgene was generated by inserting the full-length Slmap ORF downstream of the SKA promoter (59). The construct contains a downstream human growth hormone ploy(A) signal. Transgenic mice were generated as previously described (60). Mice were genotyped using primers specific to the SKA promoter and the coding region of Slmap (Supplemental Table 2).

Grip-strength testing. Six-week-old mice were lifted by the tail, causing each mouse to grasp the pull-bar assembly connected to the grip-strength meter (Columbus Instruments). The mouse was drawn along a straight line leading away from the sensor until the grasp was broken, at which time the peak force in grams was recorded. Each trial was repeated 4 times, and all measurements were performed in a blinded fashion.

Cell culture, transfection, and luciferase reporter assays. COS-7 cells (CRL-1651, ATCC) and 293T cells (CRL-3216, ATCC) were grown and maintained in DMEM containing 10% FBS. FuGENE 6 Transfection Reagent (Promega) was used for all transfections according to the manufacturer’s protocol. For luciferase assays, cells were plated onto 6-well dishes and transfected with appropriate plasmids for 48 hours. Total plasmid DNA content was kept constant by adding an empty pcDNA3.1 plasmid when necessary. For all conditions, 330 ng of each plasmid DNA was used, and 100 ng of a CMV promoter-driven LacZ expression plasmid was included in all transfections as an internal control. All experiments were performed in triplicate and were repeated twice.

Promoter cloning and site-directed mutagenesis. A 2-kb fragment of the Klhl31 promoter was cloned from mouse tail genomic DNA into a pGL3b luciferase reporter (Promega). PCR-based site-directed mutagenesis of the promoter was performed using a Quick-Change Lightning Site-Directed Mutagenesis Kit according to the manufacturer’s guidelines (Agilent Technologies). Mutations were confirmed by DNA sequencing.

Western blot analysis. Lysates were prepared by pulverizing flash-frozen tissue. Tissue powder was homogenized in RIPA buffer (Sigma-Aldrich) with the addition of protease inhibitors (Complete ULTRA Mini
Tablet) on ice in a glass dounce homogenizer. Protein concentrations were determined using a BCA protein assay kit (Ference). Samples were separated on Any kD tris-glycine buffered polyacrylamide gels (Bio-Rad) and transferred onto Immobilon P membranes (Millipore). Membranes were blocked for 1 hour at room temperature with 5% nonfat dry milk in TBST, and primary antibody hybridization was carried out overnight at 4°C using the following antibodies: Klhl31, 1:100 (sc-132316, Santa Cruz Biotechnology Inc.); Ckap4, 1:1000 (A302-257A-T, Bethyl); Slmap, 1:1000 (A304-505A-T, Bethyl); U2smg5, 1:2000 (17716-1-AP, ProteinTech); Mybpc2, 1:1000 (SAB2108180, Sigma-Aldrich); FlnC, 1:1000 (ab180941, Abcam); Gapdh, 1:10,000 (MAB374, Millipore); Myc 1:1000 (SAB2108180, Sigma-Aldrich); Flag, 1:1000 (F3165, Sigma-Aldrich); Flag-HRP, 1:2000 (A8592, Sigma-Aldrich), and Tubulin, 1:5000 (T6199, Sigma-Aldrich). Following overnight incubation in primary antibodies, the membranes were thoroughly washed in TBST and then incubated in HRP-conjugated secondary antibodies (Bio-Rad) at 1:2,000 for 1 hour of shaking at room temperature. Membranes were then developed with Western blotting luminol reagent (Sant Cruz Biotechnology Inc.) or LumiLight PLUS (Roche) for Klhl31 and exposed to autoradiograph film.

**Histology, immunofluorescence, and electron microscopy.** Following excision, skeletal muscle tissues were embedded in a mixture of OCT (Fisher) and gum tragacanth (Sigma-Aldrich) and flash-frozen in liquid nitrogen. Protein samples were collected in an Eppendorf tube on ice. Cells were vortexed at high speed for 15 seconds to facilitate the lysis. Lysates were incubated on ice for 20 minutes, then centrifuged at 23K g for 20 minutes. 700 μl of the supernatant was removed and transferred to a new ice-cold Eppendorf tube. The lysate was diluted by adding an additional 700 μl of ice-cold IP buffer. 1 ml of the diluted lysate was transferred to a new Eppendorf tube, and 50 μl of washed EZview Red anti-myc or anti-flag beads (Sigma-Aldrich) was added to the lysate and rotated overnight at 4°C. The next day, the beads were centrifuged at 8K g for 2 minutes, supernatant was removed, and beads were washed 2x with IP buffer. Following the last wash, IP buffer was removed, 40 μl of 2x Laemmli sample buffer (Bio-Rad) was added, and beads were boiled for 3 minutes. Beads were centrifuged at 8K g, and the supernatant was used for Western blot analysis (see Western blot analysis).

**Statistics.** Values are given as SEM or SD. Differences between 2 groups were assessed using unpaired 2-tailed Student’s t tests. P < 0.05 was regarded as significant. Statistical analysis was performed in Prism 7 (GraphPad).

**Study approval.** All animal procedures were approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center.

**Author contributions** JBP performed the majority of the experiments, analyzed the data, and wrote the paper. GAG performed an initial characterization of the mouse model, SB assisted in experimental studies, JRM generated the knockout and transgenic mouse lines, and RBD, NL, and ENO directed the study and helped write the paper.

**Acknowledgments** We thank Robyn Leidel for assistance and advice of the University of Texas Southwestern Electron Microscopy Core Facility, Andrew Lemoff for advice with the MS analysis, and James Richardson and Dennis Burns for help with histology and imaging. We are grateful to M. Arthur Moseley, Erik Soderblom, and the Duke University School of Medicine for the use of the Proteomics and Metabolomics Shared Resource. We would also like to thank Laura Ingle and Eric Plautz of the University of Texas Southwestern Neuro-Models Facility for grip-strength testing. We are very grateful to Jose Cabrerra for assistance with graphics. This work was supported in part by grants from the NIH (DK-099653, AR-067294, HL-130253 and HD-087351) and the Robert A. Welch Foundation (grant 1-0025 to ENO). NL was supported by a Beginning-Grant-In-Aid (13BGA17150004) from the American Heart Association. JBP was supported by a Ruth L. Kirschstein NRSA F32 NIH training grant (SF32HL123323-03).

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The Journal of Clinical Investigation


