Myopathy associated LDB3 mutation causes Z-disc disassembly and protein aggregation through PKCα and TSC2-mTOR downregulation

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Mechanical stress induced by contractions constantly threatens the integrity of muscle Z-disc, a crucial force-bearing structure in striated muscle. The PDZ-LIM proteins have been proposed to function as adaptors in transducing mechanical signals to preserve the Z-disc structure, however the underlying mechanisms remain poorly understood. Here, we show that LDB3, a well-characterized striated muscle PDZ-LIM protein, modulates mechanical stress signaling through interactions with the mechanosensing domain in filamin C, its chaperone HSPA8, and PKCα in the Z-disc of skeletal muscle. Studies of Ldb3Ala165Val/+ mice indicate that the myopathy-associated LDB3 p.Ala165Val mutation triggers early aggregation of filamin C and its chaperones at muscle Z-disc before aggregation of the mutant protein. The mutation causes protein aggregation and eventually Z-disc myofibrillar disruption by impairing PKCα and TSC2-mTOR, two important signaling pathways regulating protein stability and disposal of damaged cytoskeletal components at a major mechanosensor hub in the Z-disc of skeletal muscle.
Highly ordered arrangement of actin and myosin filaments in sarcomeres produces physical force in striated muscle. To this end, the Z-disc is of prime importance, anchoring actin filaments from adjoining sarcomeres. Striated muscle fibers are constantly exposed to strong mechanical stress. The Z-disc protein assemblies together with the dystrophin-associated glycoprotein complex and the integrin complex in the sarcolemma play essential roles in withstanding the extreme mechanical force generated during muscle contraction. Genetic defects in the components of each of these large protein complexes lead to degenerative muscle diseases, indicating the importance of these interconnected systems for the muscle integrity and function.

Mutations in genes encoding many of the Z-disc proteins have been found to cause myofibrillar myopathies (MFM), which are characterized by primary dissolution of myofibrils near the Z-disc and accumulation of degraded proteins in the sarcoplasm of muscle fibers. The dominant p.Ala165Val mutation in exon 6 of the LIM domain-binding 3 gene (Ldb3; HGNC 15710; rs121908334; NM_001080114.2:c.494 C > T, NP_001073583.1:(p.Ala165Val)) has been reported in several unrelated families of European ancestry. Whether this mutation is interpreted as likely benign (https://varsome.com/variant/hg38/rs121908334), its penetrance in the long-studied Markesbery-Griggs pedigree is 100% by age 60 years and molecular studies of six unrelated families indicated a founder mutation with a common ancient ancestry. Affected individuals present with adult-onset muscle weakness predominantly affecting the calf muscles and cardiomyopathy is usually very late and only seen in a minority of patients, which may be due to extremely low expression of LDB3 isoforms containing the exon 6 in heart.

LDB3 (ZASP; Cypher) is a highly conserved PDZ-LIM protein that plays an essential but as yet undefined role in maintaining the Z-disc integrity in contracting muscle fibers in flies, zebrafish, and mice. The PDZ domain interacts with structural Z-disc proteins and the LIM domains bind to and are phosphorylated by protein kinase C (PKC) isoforms. Alternative splicing of LDB3 is known to generate three major isoforms in skeletal muscle. The shorter isoform lacks the C-terminal LIM domains. The longer isoforms with LIM domains either contain exon 10 or exclude it (LDB3-L and LDB3-LΔex10, respectively). LDB3-L is replaced by LDB3-Lαex10 in skeletal muscle during postnatal development. Gene deletion studies in mice showed that the longer isoforms, but not LDB3-S, are important for Z-disc integrity in striated muscle. Moreover, the p.Ala165Val mutation in LDB3-Lαex10, but not other isoforms, causes F-actin disruption in transfected muscle cells. The actin-binding domain of LDB3 is mutated in MFM, but recombiant mutant proteins are correctly folded and show unaffected actin-binding affinity and kinetics. Over-expression of the LDB3-LΔex10-p. Ala165Val via intramuscular injection leads to MFM-like pathology in mouse tibialis anterior muscle fibers. However, short-term, variable, and heterogeneous expression of mutant protein in electroporated muscle fibers limited optimal investigation of disease mechanisms. Whereas knockout and cardiac-specific models have helped to characterize some LDB3 functions, the murine models of LDB3-MFM have not yet been reported and the LDB3 interactions relevant to the MFM phenotype remain unknown.

In this study, a heterozygous knock-in of the p.Ala165Val mutation in mouse Ldb3 gene closely recapitulated the genetic mutation in patients and allowed physiological levels of LDB3 isoforms in muscle tissues. The Ldb3Ala165Val/+ mice developed muscle weakness and classic MFM pathology. Our results indicate that LDB3 acts as a signaling adapter in a major mechanosensor assembly through interactions with filamin C, its chaperone HSPA8, and PKCa at skeletal muscle Z-disc. The LDB3 p. Ala165Val mutation impairs PKCa and TSC2-mTOR mediated homeostasis in this large protein assembly leading to protein aggregation myopathy.

**Results**

Generation of Ldb3Ala165Val/+ knock-in mice. We introduced the p.Ala165Val point mutation (chr14:34571772 C > T; GRCh38/mm10; C57BL/6 N), responsible for MFM, into exon 6 of the endogenous mouse Ldb3 gene to generate Ldb3Ala165Val/+ mice by homologous recombination (Fig. 1a; Supplementary Fig. 1a). The mutated residue is conserved, and overall amino acid identity is >92% for LDB3 isoforms between human and mouse. Targeted gene sequencing and Southern blot analysis confirmed the accuracy of gene editing and the absence of other mutations in the recombinant region (Supplementary Fig. 1b). The presence of the NP_001034164.1:(p.Ala165Val) mutation was further validated by Sanger sequencing (Fig. 1b). The levels of Ldb3 mRNA and that of the major LDB3 protein isoforms in the vastus muscle of Ldb3Ala165Val/+ mice were similar to Ldb3+/+ littermates (Fig. 1c; Supplementary Fig. 1c; Supplementary Data 1), suggesting that the point mutation is not affecting transcript or protein stability in muscle tissue. Mating of Ldb3Ala165Val/+ mice resulted in 26% Ldb3+/+, 54% Ldb3Ala165Val/+ and 19% Ldb3Ala165Val/Ala165Val mice (n = 84; 10 litters), indicating that the mutation did not affect mouse viability.

Ldb3Ala165Val/+ mice develop progressive muscle weakness. We screened Ldb3Ala165Val/+ mice and Ldb3+/+ littermates at 3, 6, and 9 months of age for signs of muscle weakness. The mice had normal locomotor coordination assessed by Rotarod. Grip strength tests showed a significant decline in the Ldb3Ala165Val/+ mice compared with Ldb3+/+ littermates at 3, 6, and 9 months of age (Fig. 1d; Supplementary Data 2). A two-way analysis of variance (ANOVA) with post hoc Bonferroni correction yielded significant effects of genotype, F(1, 56) = 63.3, p < 0.0001 and age, F(2, 56) = 32.9, p < 0.0001, as well as the age and genotype interaction, F(2, 56) = 6.5, p = 0.003. We found that the reduced grip strength was associated with decreased specific isometric force of the extensor digitorum longus muscle in 6-month-old Ldb3Ala165Val/+ mice compared with that in Ldb3+/+ littermates (Fig. 1e; Supplementary Data 3). A two-way ANOVA with post hoc Bonferroni correction yielded a significant effect of genotype F(1, 56) = 17.93, p = 0.008. Mean physical impulse to hold the wire grid reflecting the total sustained force exerted to oppose the gravitational force was decreased by about 60% in 9-month-old Ldb3Ala165Val/+ mice compared with that in Ldb3+/+ littermates (Fig. 1f; Supplementary Data 4).

LDB3 p.Ala165Val mutation causes a classic pathological MFM phenotype in mouse skeletal muscle. Skeletal muscle histology was assessed at 4, 6, and 8 months of age in the hindlimbs muscles of Ldb3Ala165Val/+ mice and their Ldb3+/+ littermates. Muscle histology in mutant mice at 4 months of age was similar to that in wildtype mice (Fig. 2a). In contrast, Gomori trichrome staining of transverse sections of the soleus muscle of 8-month-old Ldb3Ala165Val/+ mice showed dark blue to blue-red hyaline granular deposits and rimmed vacuoles in the sarcoplasm of muscle fibers (Fig. 2a). These abnormal fibers showed decrease in oxidative enzyme activity in the region of protein deposits and increased enzyme activity mostly in the periphery, suggesting abnormal mitochondrial function. In addition, non-specific myopathic changes such as increases in internal nuclei, hypertrophic fibers, and muscle fibers with rounded contour were observed in the vastus and tibialis anterior muscles of mutant mice (Fig. 2b). To quantitate the changes in myonuclear location, we

**Figures and Supplemental Information**
performed morphometry with wheat germ agglutinin (WGA) to outline muscle membrane and distinguished muscle nuclei. Relative to wildtype littermates, Ldb3Ala165Val/+ mice had a significantly higher percentage of fibers with internal nuclei in the tibialis anterior muscle (p < 0.01; Supplementary Fig. 2a). Muscle fiber type distribution was unchanged in the soleus and vastus muscles of Ldb3Ala165Val/+ mice (Supplementary Fig. 2b–d). Muscle fiber size distribution for types I and IIA in Ldb3Ala165Val/+ mice was shifted towards right compared to those from Ldb3+/+ mice, whereas distribution of type IIB fiber size was unchanged (Supplementary Fig. 2e). Mean minimal Feret’s diameter for fiber types I and IIA was higher in Ldb3Ala165Val/+ mice relative to wildtype mice, but the change was not statistically significant in the Bonferroni’s multiple comparison test (Supplementary Fig. 2f). Accordingly, variance coefficient of the minimal muscle fiber diameter was unchanged for all fiber types in Ldb3Ala165Val/+ mice (Supplementary Fig. 2g).

Immunostaining studies showed sarcoplasmic accumulations of the MFM-associated Z-disc proteins including LDB3, myotilin, desmin, filamin C, and αB-crystallin, as well as ubiquitin in same fibers of 8-month-old Ldb3Ala165Val/+ mice (Fig. 2c). Counts of a mean of 498 fibers in transverse soleus muscle section of five mutant mice each showed a mean of 64% and 44% muscle fibers contained filamin C and ubiquitin accumulations, respectively (Supplementary Fig. 3a). These protein accumulations occurred in multiple or diffuse form in muscle sarcoplasm. The abnormal fibers were distributed focally surrounded by muscle fibers without protein aggregation. Such protein aggregates were not observed in Ldb3Ala165Val/+ mice. Electron microscopy studies showed the Z-disc myofibrillar disruption, dislocated enlarged mitochondria, and autophagic vacuoles in the soleus muscle fibers of 6-month-old Ldb3Ala165Val/+ mice and normal Z-disc and sarcomere architecture in their Ldb3+/+ littermates (Fig. 2d–f).

LDB3Ala165Val protein triggers early aggregation of filamin C and its chaperones, eventually leading to aggregation of the mutant protein and Z-disc myofibrillar disruption. Patients usually present with advanced stages of myopathic disease at the time of biopsy, thereby limiting studies of early pathological features in the MFM. We examined the early cellular and molecular events that lead to the MFM pathology in Ldb3Ala165Val/+ mice. We found filamin C aggregates in muscle fibers that had normal...
LDB3 immunostaining in transverse vastus and soleus muscle sections of 6-month-old Ldb3<sup>Ala165Val/+</sup> mice (Fig. 3a). Counts of a mean of 623 fibers from vastus muscle transverse sections of five mutant mice each showed a mean of 15% fibers contained filamin C accumulations (Supplementary Fig. 3b). Such protein aggregates were not seen in wildtype littermates. The filamin C accumulations were more prominent compared to LDB3 in the vastus lateralis muscle fibers of patients sharing the same mutation (Fig. 3a). Filamin C, an MFM gene product<sup>23</sup>, acts as a primary mechanosensitive crosslink for actin filaments at the Z-discs<sup>24</sup>. Mechanical strain on the actin network results in unfolding of the filamin C crosslinks, which are degraded through the conserved tension-induced chaperone-assisted selective autophagy (CASA) pathway<sup>25</sup>. Immunostaining of serial muscle transverse sections showed that filamin C aggregates were accompanied with the CASA chaperones BAG3, HSPA8, HSPB8, and ubiquitin in muscle fibers of Ldb3<sup>Ala165Val/+</sup> mice but not in their Ldb3<sup>+/+</sup> littermates (Fig. 3b, c). These sarcoplasmic protein aggregations were also found in the vastus muscle fibers of MFM patients with the LDB3 p.Ala165Val mutation (Fig. 3d).

Serial longitudinal tibialis anterior muscle sections of 4- and 8-month-old Ldb3<sup>+/+</sup> mice showed normal Z-disc distribution of
LDB3, lamin C, myotilin, BAG3, and HSPA8 proteins (Fig. 4a and f). Immunostaining of the tibialis anterior muscle sections in 4-month-old Ldb3Ala165Val/+ mice showed aggregates of filament C, BAG3, and HSPA8 co-localizing at the Z-discs of same muscle fibers, whereas staining for LDB3 and myotilin was normal (Fig. 4b–e). Co-aggregation of LDB3 with filament C, myotilin, BAG3, and HSPA8 with or without Z-disc myofibrillar disruption occurred at a later disease stage in 8-month-old Ldb3Ala165Val/+ mice (Fig. 4g–j). Prominent accumulations of the CASA chaperones were found in muscle fibers harboring filament C and other myofibrillar protein aggregates but not in adjoining fibers without protein aggregation (Figs. 3c, d and 4d, e, i, j). These results indicate that the LDB3 p.Ala165Val mutation causes aggregation of damaged filament C before LDB3 and other MFM proteins, eventually leading to Z-disc myofibrillar disruption in skeletal muscle. Muscle fibers react to ubiquitinated protein aggregation with accumulations of the CASA chaperones, but the protein degradation systems are not efficient in clearing the damaged protein aggregates.

**LDB3**WT and LDB3**Ala165Val** proteins interact with mechanosensing domain in filament C and HSPA8, a chaperone in tension-induced autophagy pathway. We found early and prominent filament C aggregates in skeletal muscle fibers of Ldb3Ala165Val/+ mice, indicating that the LDB3 p.Ala165Val mutation likely affects the stability or degradation of filament C. Immunoblotting studies showed that whole muscle levels of filament C and the CASA chaperones were not altered in skeletal muscle of Ldb3Ala165Val/+ mice (Fig. 5a). The association of modular protein-interacting domains enables LDB3 to serve as an adapter protein that recruits multiple proteins to a localized site of action in a subcellular domain. To this end, we conducted a yeast two-hybrid (Y2H) screen of human skeletal muscle cDNA library using a human LDB3 peptide encoded by exons 8–11 as bait to identify novel interacting proteins. This bait identified 46 prey clones encoding six potential LDB3 interactors including two Z-disc associated proteins filament C and HSPA8, with strong confidence in the interaction (Fig. 5b; Supplementary Table 1).

Interestingly, the five filament C clones encoded the rod domain 2 immunoglobulin-like (Ig) 17–21 repeats, a precisely tuned mechanosensor that detects and responds to the small cytoskeletal forces during muscle contraction. In addition, three prey clones encoded an internal region of HSPA8 (aa 237–468; NM_006597.5) that is recruited by BAG3 to the Ig19–21 repeats of unfolded filament C as a part of the CASA complex forming the molecular basis for sensing force-induced unfolding. Pairwise Y2H assays showed that inclusion of the exon 10-encoded region in the bait corresponding to LDB3-L isoform abolished LDB3 binding to HSPA8 (Fig. 5c). These findings support our previous observations of isoform-specific LDB3 interactions with skeletal actin, GST pulldown assays using tagged wildtype (WT) and mutant LDB3-LΔex10 proteins as bait showed that the LDB3 p. Ala165Val mutation did not impair filament C and BAG3 interactions (Fig. 5d). Binding of WT and mutant LDB3-LΔex10 with filament C Ig 17–21 domain and HSPA8 was validated by independent co-immunoprecipitation (IP) of tagged proteins from transfected COS-7 cell lysates (Fig. 5e). Endogenous interactions of LDB3 with filament C and HSPA8 in skeletal muscle were confirmed by co-IP of these proteins from the tibialis anterior muscle myofibrillar fraction of 4-month-old Ldb3Ala165Val/+ mice and Ldb3Δ/+ littermates (Fig. 5f). These findings point to mechanosensing roles of LDB3 through interactions with filament C and HSPA8 in the Z-disc. The p.Ala165Val mutation does not appear to affect these LDB3 interactions in skeletal muscle.

**LDB3**Ala165Val protein impairs PKCa and TSC2-mTOR signaling in skeletal muscle. Previous studies suggested that LDB3 can act as a signaling scaffold to regulate protein function. To identify altered signaling pathways leading to MFM pathology in Ldb3Ala165Val/+ mice, a reverse-phase protein array (RPPA) was done on the vastus muscle lysates of 4- and 8-month-old Ldb3Ala165Val/+ mice and their gender-matched Ldb3Δ/+ littermates. It should be noted that filament C aggregates were observed in skeletal muscle fibers of 4-month-old mutant mice (Fig. 4b, d), whereas Z-disc myofibrillar disruption is evident after 6 months of age (Figs. 2c and 4g–i). RPPA measured changes in the abundance and post-translational modifications of proteins using 248 antibodies. The analysis showed a significant down-regulation of the LDB3 interactor PKCa, as well as the most important negative mTOR regulator TSC2 in the vastus muscle of 4-month-old Ldb3Ala165Val/+ mice (≥1.5-fold, corrected p ≤ 0.05; Fig. 6a; Supplementary Table 2a; Supplementary Data 6). Levels of PKCa and TSC2 were downregulated by 1.7 and 1.5-fold, respectively in the vastus muscle of 4-month-old Ldb3Ala165Val/+ mice compared to Ldb3Δ/+ littermates. PKCa levels were also downregulated by 1.8 fold in the vastus muscle of 8-month-old mutant mice relative to wildtype littermates (Fig. 6b; Supplementary Table 2b; Supplementary Data 7). TSC2 showed a downward trend in the older mutant mice (1.4 fold, corrected p = 0.07). Validation of the RPPA results was done by immunoblotting of the vastus muscle lysates of Ldb3Ala165Val/+ mice and...
Fig. 3 Immunolocalization of filamin C and CASA chaperone complex in skeletal muscle of Ldb3Ala165Val/+ mice and patients sharing the same mutation. a Representative immunofluorescence on frozen vastus lateralis and soleus muscle transverse section of 6-month-old Ldb3Ala165Val/+ mice (n = 9 and 5, respectively) and Ldb3+/+ littermates (n = 5) and an MFM patient (n = 3) with the LDB3 p.Ala165Val mutation stained with filamin C and LDB3 antibodies. White arrows indicate muscle fibers with sarcoplasmic filamin C aggregates that have normal sarcoplasmic LDB3 distribution in Ldb3Ala165Val/+ mice and relatively less extensive LDB3 accumulations in the patient. b–d Representative immunofluorescence on frozen soleus muscle serial transverse sections of 8-month-old Ldb3+/+ mice (b; n = 6) and Ldb3Ala165Val/+ littermates (c; n = 8), and the vastus lateralis muscle of patient (d; n = 3) stained with filamin C, BAG3, HSPA8, HSPB8, and ubiquitin antibodies. White arrows indicate same muscle fibers with sarcoplasmic accumulations of filamin C and the CASA proteins in Ldb3Ala165Val/+ mice and patient. Such protein aggregates are not seen in the sections obtained from Ldb3+/+ mice. Scale bars = 50 μm.
Ldb3+/+ littermates. Whole muscle levels of PKCα in 4- and 8-month-old Ldb3Ala165Val/+ mice were decreased to about 50% of those in Ldb3+/+ littermates, and levels of TSC2 in the mutant mice were reduced to almost two-thirds of those in Ldb3+/+ mice (Fig. 6c–f; Supplementary Data 8, 9). Downregulation of PKCα and TSC2 indicates a likely mechanism for the observed aggregation of damaged filamin C in muscle fibers of Ldb3Ala165Val/+ mice as PKCα stabilizes filamin C in muscle Z-disc through phosphorylation34, and TSC2 initiates CASA-mediated degradation of damaged filamin through local mTORC1 inhibition35.

**Discussion**

Here, we unveil a previously undescribed mechanism by which a disease-associated mutation in LDB3, a PDZ-LIM protein, causes protein aggregation and myofibrillar Z-disc disruption in skeletal muscle. Our data demonstrate that LDB3 directly binds to the mechanosensing domain of filamin C, and its chaperone HSPA8, a central player in protein folding and proteostasis control. Studies in knock-in mice show that the MFM-associated LDB3 p.Ala165Val mutation causes early aggregation of filamin C and its chaperones in the tension-induced CASA pathway before...
Fig. 4 Progressive protein aggregation and Z-disc myofibrillar disruption in skeletal muscle of Ldb3Ala165Val/+ mice. Representative immunofluorescence staining on perfused tibialis anterior muscle consecutive longitudinal sections of 4-month-old (a–e) and 8-month-old (f–j) Ldb3Ala165Val/+ mice and their D3+/− littermates. a Muscle sections of 4-month-old Ldb3−/− mice (n = 4) show normal Z-disc staining for LDB3, filamin C, myotilin, BAG3, and HSPA8 proteins. b–c Muscle sections of 4-month-old Ldb3Ala165Val/+ mice (n = 6) show same muscle fiber co-stained with LDB3 and filamin C antibodies (b), and LDB3 and myotilin antibodies (c). d–e Muscle sections of 4-month-old Ldb3Ala165Val/+ mice (n = 6) show same muscle fiber co-stained with filamin C and HSPA8 antibodies (d), and BAG3 and HSPA8 antibodies (e). Filamin C, HSPA8, and BAG3 aggregates are seen at the Z-disc spanning multiple sarcomeres in same fiber. In contrast, the LDB3 and myotilin antibodies show normal Z-disc staining in the fiber with filamin C aggregates. f Muscle sections of 8-month-old Ldb3+/− mice (n = 5) show normal Z-disc staining for LDB3, filamin C, myotilin, BAG3, and HSPA8 proteins. Top and bottom panel each shows same muscle fiber co-stained with LDB3 and filamin C antibodies (g), and LDB3 and myotilin antibodies (h) in muscle sections of 8-month-old Ldb3Ala165Val/+ mice (n = 7). Muscle sections of 8-month-old Ldb3Ala165Val/+ mice (n = 7) show same muscle fiber co-stained with LDB3 and BAG3 antibodies (i), and LDB3 and HSPA8 antibodies (j). LDB3 aggregates colocalize with filamin C, myotilin, BAG3, and HSPA8 at the Z-discs in muscle fibers. The protein aggregates are seen in muscle fibers with normal periodicity of the Z-discs and with disorganized Z-disc periodicity indicating Z-disc myofibrillar disruption. A part of adjacent muscle fiber with normal staining pattern is shown at bottom in each image for comparison. Scale bar = 10 μm and applies to all images.

Fig. 5 Characterization of LDB3 interactions with filamin C and HSPA8. a Representative immunoblots showing protein levels of filamin C, BAG3, and HSPA8 relative to β-actin in the vastus muscle of 8-month-old Ldb3Ala165Val/+ mice and Ldb3−/− littermates. Data represent n = 5 mice per group and triplicate assays. b Schematics of LDB3 interaction with the mechanosensing domains Ig17–21 of filamin C and its chaperone HSPA8 as identified by yeast two-hybrid (Y2H) screen of a human skeletal muscle cDNA library. See Supplementary Table 1. Locations of the LDB3 bait encoded by exons 8–11Δ10 (yellow) and the p.Ala165Val mutation (white; asterisk) within actin-binding domain (ABD; blue) are shown in LDB3-LΔ10 isoform. Domain composition of the prey clones are shown. c Pairwise Y2H assays demonstrating interaction between LDB3 peptides and HSPA8. Positive interactions show yeast growth on the media deficient in HIS3 and ADE2. Yeast cells co-transformed with empty bait vector and the HSPA8 prey show no growth (labeled −). Transformation efficiency was uniform for all constructs. Sequential tenfold yeast dilutions are shown. d GST pulldown assay shows that GST-tagged wildtype (WT) and mutant LDB3-LΔex10 (Ala165Val) but not GST alone pulled down filamin C and its chaperator BAG3 from the vastus muscle lysates of wildtype mice. Data represent n = 3 mice per triplicate assays. e Co-immunoprecipitation (co-IP) assays show that a FLAG antibody pulled down the FLAG-tagged WT and mutant (Ala165Val) LDB3-LΔex10 together with HA-tagged filamin C rod domain Ig17–21 and HSPA8 in Cos7 cells. Data represent triplicate assays. f The proteins were detected with anti-FLAG and anti-HA antibodies. f Co-IP assays show that an LDB3 antibody pulled down LDB3 isoforms together with filamin C and HSPA8 from the tibialis anterior muscle lysates of Ldb3Ala165Val/+ and Ldb3−/− mice. Data represent n = 3 mice per group and triplicate assays.

aggregation of the mutant LDB3 protein in muscle fibers. The LDB3 p.Ala165Val mutation destabilizes filamin C and stalls the removal of damaged filamin C through impaired PKCa and TSC2-mTOR signaling at arguably the most important mechanosensor hub in the Z-disc of skeletal muscle (summarized in Fig. 7). The presence of chaperones and ubiquitin in these protein aggregates likely represents impairment of protein degradation pathways. Further, pathology studies show that muscle fibers with protein aggregates develop the Z-disc myofibrillar disruption in skeletal muscle of Ldb3Ala165Val/+ mice.

LDB3 is well-poised to play a role in mechanosensor function in striated muscle. Homozygous LDB3-null mice display fragmented Z-discs in the diaphragm muscle after birth, but not during embryonic development[1]. Since the diaphragm does not
vinculin in the vastus muscle of 4-month-old levels in the vastus muscle lysates of 4 months (PKC). This is supported by that the LDB3 interactions through this domain likely play role in sensing external tension in striated muscle. LDB3 interacts with skeletal actin and is uniquely present in the postnatal longer isoform. Previously we have shown that the LDB3 p. Ala165Val mutation in the postnatal longer LDB3 isoform, but not other isoforms, leads to myofibrillar disruption, suggesting that toxicity is not through altered ligand binding properties of mutant protein. The p.Ala165Val mutation leading to the MFM phenotype, indicating that most of the disease pathways converge onto a final pathway centered on the large LDB3-filamin C-CASA chaperone scaffold for this diverse group of myopathies. These findings underscore critical roles for LDB3 in recruiting key proteins to strategic sites and facilitating mechanosensing in striated muscle.

LDB3 is known to anchor PKC isozymes at the Z-disc of striated muscle. PKCa accounts for 97% of the conventional PKC function in skeletal muscle. We found a significant decrease (~50%) in PKCa expression in skeletal muscle of Ldb3^Ala165Val/+ mice, before the MFM phenotype. Mechanisms by which the MFM-associated LDB3 p.Ala165Val mutation downregulates PKCa in skeletal muscle are yet unknown, but presumably the mutation affects ligand binding, phosphorylation, or autoinhibition of the kinase that effectively leads to its downregulation. The longer LDB3 isoforms have been shown to negatively regulate the PKCa expression in heart. To this end, the p.Ala165Val mutation likely acts as a gain-of-function mutation leading to the MFM phenotype. Interestingly, PKCa has been viewed as an attractive therapeutic target in many diverse cancers and degenerative diseases of heart and brain. Loss-of-function mutations in PKCa are prevalent in cancers, and studies in cancer cell lines showed that small changes in the PKC expression have large effects on cellular functions. It is known that PKCa phosphorylates filamin C and prevents its calpain-mediated proteolysis, thus preserving its dimerization function.

Mutations in many proteins in this interactome, such as LDB3, filamin C, myotilin, BAG3, DNAJB6, and HSPB8 lead to MFM phenotype, indicating that most of the disease pathways converge onto a final pathway centered on the large LDB3-filamin C-CASA chaperone scaffold for this diverse group of myopathies. These findings underscore critical roles for LDB3 in recruiting key proteins to strategic sites and facilitating mechanosensing in striated muscle.
proteins. Taken together, reduction in PKCα likely predisposes filamin C to calpain-mediated proteolysis leading to protein aggregation in skeletal muscle of Ldb3<sup>3Ala165Val/+</sup> mice (Fig. 7c).

The aggregation of damaged filamin C aggravates the autophagy pathway in muscle fibers of Ldb3<sup>3Ala165Val/+</sup> mice. This is evidenced by marked accumulations of the CASA chaperones in the muscle fibers containing protein aggregates and increased levels of the autophagy markers after colchicine blockage in skeletal muscle of 4-month-old mice. However, it appears that the strain on the cellular degradation system ultimately limits the capacity of the CASA chaperones to remove the damaged misfolded proteins and causes myopathy. This connection is supported by the hallmark MFM pathology changes in the Ldb3<sup>3Ala165Val/+</sup> mice that resemble muscle pathology caused by mutations in filamin C and CASA components BAG3, DNAJB6, and HSPB8, which have been proposed to negatively affect CASA function. The CASA pathway constantly operates at the Z-disc and removes mechanically damaged proteins such as filamin C, thus it differs from the atrophy-driven autophagy pathways. Recruitment of TSC1:TSC2 – mTORC1 assemblies by BAG3 to skeletal muscle Z-disc is required for the disposal of damaged filamin C through the CASA pathway. Within the complex, TSC1 stabilizes TSC2, whereas TSC2 acts as a GTPase activating protein and integrates signals from various kinases leading to mTORC1 regulation in the cell. It is possible that the reduction of TSC2 in skeletal muscle of Ldb3<sup>3Ala165Val/+</sup> mice perturbs CASA function, leading to filamin C aggregation in muscle fibers (Fig. 7c). In addition, chronic changes in PKCα and TSC2-mTORC1 levels likely affect the balance of lysosomal autophagy in skeletal muscle of Ldb3<sup>3Ala165Val/+</sup> mice.
In Ldb3Ala165Val/+ muscle, the decrease in PKCα levels may be related to reduced TSC2 through mTORC2 inhibition. PKCα is one of the best-characterized substrates of TSC2-mTORC2 that potentially regulates subcellular localization of mTORC1 and mTORC2 components, thereby permitting selective activation of specific targets. It remains to be determined whether the MFM-associated mutation affects subcellular translocation of mTORC complexes to the Z-disc and protects these proteins from proteolysis by calpains. The CASA pathway constantly operates at the Z-disc mediating degradation of large cytoskeleton components including filament C damaged during mechanical strain. The CASA activity depends on local mTORC1 inhibition through BAG3-recruited TSC2-TSC1 signaling. PKCα is modulated by TSC2-mTORC2 signaling and the kinase may regulate mTOR assemblies including spatial localization (dashed lines). The Ldb3Ala165Val downregulates PKCα and TSC2-mTORC2, two signaling proteins monitoring the integrity of the Z-disc assembly. Decreased PKCα promotes proteolysis by calpains leading to aggregation of damaged proteins. Reduced TSC2-mTORC together with increased strain on capacity of degradation pathway leads to impaired CASA function aggravating damaged protein aggregation, eventually leading to the Z-disc disassembly and myofibrillar disruption.

**Methods**

**Mice.** Wildtype mice (C57BL/6 N) were from Charles River laboratories (Wilmington, MA). The β-actin-cre mice were described previously. Heterozygous Ldb3-null mice (C57BL/6N - Atn1Brd/a Ldb3ΔflΔfl;C57BL/6N) were from the Mutant Mouse Resource & Research Centers. All animals were housed in the animal care unit of the NINDS according to the NIH animal care guidelines. All animal studies were authorized by the Institutional Animal Care and Use Committee of the NINDS.

**Antibodies.** Primary antibodies are listed in Supplementary Table 3. Antibodies against LDB3 were generated by immunizing rabbits with a peptide corresponding to amino acid residues 116–130 encoded by exon 6 of human LDB3 (NP_001075395). Secondary antibodies are listed in Supplementary Table 4. Primary antibodies are listed in Supplementary Table 3. Antibodies against LDB3 were generated by immunizing rabbits with a peptide corresponding to amino acid residues 116–130 encoded by exon 6 of human LDB3 (NP_001075395). Secondary antibodies are listed in Supplementary Table 4. Primary antibodies are listed in Supplementary Table 3.

**DNA constructs.** Primers are listed in Supplementary Table 4. A fragment encoding the rod domain 2 Ig17-21 of human lamin C (nucleotides 5629-7352) was cloned into pCMV-HA (Clontech). Full-length human HSPA8 cDNA (I.M.A.G.E clone ID NM_001080116) and pGBKT7-LDB3 exons 8-10-11 and 8-11 were amplified by PCR with cloned into pCMV-HA vector. Constructs for FLAG-tagged human skeletal muscle WT and Ala165Val Ldb3+/- mice (Supplementary Fig. 5b–d). Alexa Fluor, IRDye, and HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies were purchased from Invitrogen, LI-COR biosciences, and Jackson Immunoresearch Laboratories, respectively.

**Generation of Ldb3Ala165Val/+ mice.** Strategy for generating the p-Ala165Val knock-in mutation in the mouse Ldb3 gene is shown in Supplementary Fig. 1a. The RP23-244C16 Bacterial Artificial Chromosome (BAC) clone containing the mouse Ldb3 gene (C57BL/6 N) was modified to reproduce the MFM causative p. (chr1:43547772 C > T; GRCh38/mm10; C57BL/6 N), by a galK-selected recombining in SW102 cells, as described previously. The targeting construct homologous to the region flanking the exon 6 mutation was generated by PCR using two 100-mer oligonucleotides with 20 bp overlap at the 3' end. Next, the Ldb3 genomic region between introns 3 and 8 (chr1:434576235–4378097) containing the mutation was retrieved from the modified BAC clone PL233 gene-targeting vector by “gap repair”, as described previously. And finally, a neomycin selecting cassette (PGK-neo), flanked by loxp sites, was inserted within intron 6 between chr1:435470741 and 435470742 base pairs in the direction of gene transcription by gap repair using PL452 vector containing ~500 bp homology arms.
flanking the insertion site, as described previously\(^6\). The modified PL253 gene-targeting vector was linearized with Not I and electroporated into embryonic stem (ES) cells generated from B6;129 hybrid mice. Targeted ES cells were identified by Southern blotting and confirmed by Sanger sequencing. Positive ES cell clones were injected into blastocysts to obtain chimeric mice as described previously\(^6\). The presence of the selection cassette disrupted allelic expression in Ldb3\_gloxed-6AAs3V1\_+/mice. Functional knockout lines were generated by using Ldb3\_gloxed-6AAs3V1\_+/mice. Functional knock-in lines were activated by crossing Ldb3\_gloxed-6AAs3V1\_+/mice with β-actin-cre transgenic mice (C57BL/6 N). Offsprings were bred with wildtype mice to remove Cre-recombinase. The resulting Ldb3\_gloxed-6AAs3V1\_+/ mice were backcrossed for at least 5 generations on C57BL/6N background.

**Targeted gene sequencing of the Ldb3 recombinant region.** Mouse genomic DNA was PCR amplified using primer sets described in Supplementary Table 4. DNA was precipitated using the Next Advance kit (Illumina). The concentrations of the indexed libraries were analyzed on the Agilent 2200 TapeStation using the D1000 Kit (Agilent Technologies). Equimolar amounts of the indexed libraries were pooled to obtain a 4 nM library mixture. After denaturation and further diluting, the final 12 pmol library was loaded into an Illumina cartridge. Sequencing was performed using the Illumina MiSeq Reagent Kit v2 (300 Cycles) on the Illumina MiSeq instrument following the manufacturer’s instructions.

**Southern blotting.** Tail DNA was extracted by digestion in buffer (1 M Tris-HCl pH 8.5, 0.5 M EDTA, 5 M NaCl, 10% SDS, 10 mg/ml proteinase K) overnight at 55 °C with continuous shaking in low speed. Lysates were cleared by centrifugation at 14,000 rpm for 5 min, DNA was precipitated from equal volume of isopropanol, pelleted at 14,000 rpm for 5 min, washed three times in 70% ethanol, air dried, and rehydrated in water. Digoxigenin-labeled DNA probes hybridized to the 5’ nucleotides 34579195–34579956; NC_000080.6 and 3’ nucleotides 34564772–34565187 flanking sequence of the recombinant Ldb3 genomic region were used. Mouse genomic DNA samples (10 μg) were digested with EcoRV (New England Biolabs) overnight at 37 °C, separated on a 0.7% agarose gel over 20 h and blotted onto a positively charged nylon membrane (Roche Diagnostic) by capillary transfer for 48 h. Hybridization was done overnight at 45 °C with agitation at 10 rpm. Membranes were washed twice with 2X SSC containing 0.1% SDS for 5 min at 65 °C and then twice with 0.1X SSC containing 0.1% SDS for 15 min at 65 °C. DIG-labeled probes after hybridization to target DNA were detected using DIG High Prime Detection kit II (Roche Diagnostic). Images were acquired on a ChemiDoc imager (BioRad) using Image Lab software (version 5.2).

**Mouse genotyping.** Mouse genotypes were identified by PCR using primers to detect the residualloxP site as well as a SNP genotyping assay on genomic DNA (Supplementary Fig. 1d, e). The presence of modification was validated at gene and genomic DNA by Sanger sequencing. Positive ES cell clones were injected into blastocysts to obtain chimeric mice. See Supplementary Data 2 and 4 for source data.

**RNA Isolation from tissue.** Mouse skeletal muscle tissue was homogenized using Amicon Trizol Reagent (Life Technologies) and RNAse-free 0.5 mm zirconium oxide beads in a Bullet Blender homogenizer (Next Advance, Troy, NY). Total RNA was precipitated using the Next Advance kit (Illumina). The concentrations of the indexed libraries were analyzed on the Agilent 2200 TapeStation using the D1000 Kit (Agilent Technologies). Equimolar amounts of the indexed libraries were pooled to obtain a 4 nM library mixture. After denaturation and further diluting, the final 12 pmol library was loaded into an Illumina cartridge. Sequencing was performed using the Illumina MiSeq Reagent Kit v2 (300 Cycles) on the Illumina MiSeq instrument following the manufacturer’s instructions.

**Histology.** Frozen 10 μm thick transverse muscle sections were stained with modified Gomori trichrome and nicotinamide adenine dinucleotide dehydrogenase-tetrazolium reductase (NADH-TR) enzymatic activity using standard procedures. Heart tissues obtained from mice were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C and then cryoprotected in 30% sucrose at 4 °C. For histological examinations, hearts were embedded into wax blocks of paraffin and 5 μm thick sections were stained with hematoxylin and eosin. See Supplementary Data 2 for source data.
Longitudinal sections (8 μm) were collected onto slides and antigen retrieval was carried out by boiling slides in citrate buffer (pH 6.0) for 10 min before immunostaining. Images were acquired on a Leica SP8 confocal microscope (TCS SP5 576 II) using a 63×/NA 1.4 oil immersion Plan-Apochromat objective and processed as described for frozen sections.

For fiber-type staining, unfixed frozen transverse soleus and vastus muscle sections (10 and 8 μm, respectively) were blocked with 10% goat serum/PBS for 1 h and then incubated at 4°C in an antibody cocktail containing myosin heavy chain (MYHC) types I, IIA, IIB, and IX (BA-F8, SC-71, BF-F3, and 6H1, respectively). Developmental Studies Hybridoma Bank; Supplementary Table 3). Fluorescence-conjugated secondary antibodies to different mouse immunoglobulin subtypes (IgG2a, Alexa Fluor 488 or 568, and IgM – Alexa Fluor 568, ThermoFisher Scientific; CY5-IgG1, Abcam) were applied for 1 h at room temperature to visualize MYHC expression. In addition, wheat germ agglutinin – Alexa Fluor 488 conjugate (WGA; ThermoFisher Scientific) was applied to stain muscle membrane for fiber type – quantization. Sections were mounted with Fluoromount – G with DAPI (Southern Biotech). Images were obtained using a Leica epifluorescent microscope (DM6000B SD) equipped with a motorized stage. Images were acquired in Leica Application Suite (LAS) X software with a 20×/NA 0.4 objective. The LAS X Navigator tool was used to take images from adjacent fields and digitally stitch them (with 10% overlap) to form a single image of the entire soleus muscle cross-section for analysis. For vastus muscle, nonoverlapping areas were photographed for analysis. Myonuclei counting, muscle fibers with sarcomplasmic protein aggregate quantitation, muscle fiber typing (using the counting tool), and minimal Feret’s diameter measurement for fiber size were performed for all fibers excluding those on the edges by two individuals using open-source image processing software Image J/Fiji (National Institutes of Health, Bethesda, MD).

Electron microscopy. Mice were transcardially perfused with 2% glutaraldehyde and 2% paraformaldehyde in PBS and skeletal muscles were post-fixed in the same buffer in 0.1 M sodium cacodylate. The tissue embedding and staining were carried out in the NINDS electron microscopy facility using a standard approach. Images were acquired on an electron microscope (JEOL 200CX, Jeol, Inc.) and processed with Adobe Photoshop Creative Cloud v2017.

Immunoblotting. Protein was extracted from transverse muscle midbelly crosssections (n = 15, 10 μm thick) using SDS buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% β-ME, 25 mM EDTA pH 8) supplemented with protease inhibitor (Roche). Equal volumes were loaded for relative protein quantification by immunoblotting. For PKC resolved on Tris-Glycine polyacrylamide gels were transferred to nitrocellulose with Adobe Photoshop Creative Cloud v2017.

For PKC immunoblotting. For PKC resolved on Tris-Glycine polyacrylamide gels were transferred to nitrocellulose with protease and phosphatase inhibitors (Roche) using polytron homogenizer. Targeting the nonoverlapping areas for analysis. Myonuclei counting, muscle fibers with sarcomplasmic protein aggregate quantitation, muscle fiber typing (using the counting tool), and minimal Feret’s diameter measurement for fiber size were performed for all fibers excluding those on the edges by two individuals using open-source image processing software Image J/Fiji (National Institutes of Health, Bethesda, MD).

Autophagosome flux. Measurement of autophagosome flux in mouse skeletal muscle tissue using lysosomal blockade by colchicine was done as described previously37. Mice were treated with intraperitoneal colchicine (0.4 mg/kg/day, Sigma) or vehicle alone (PBS pH 7.4) for 3 days. The day following the last injection, hindlimb muscles were collected for protein isolation. Levels of autophagosome markers LC3-II and sequestosome 1 (p62) were quantified by immunoblotting. GAPDH was used as a loading control. The samples were run on two gels (one mini gel could not accommodate all sample loadings) in the same electrophoresis tank. The protein transfer from both gels was done simultaneously on a single membrane which was then processed for immunoblotting. For densitometric analysis, all the band intensities were normalized to that of one PBS-treated Ldb3 +/– sample in each membrane. See Supplementary Fig. 6b for full-length blot images. Autophagosome flux values were derived by subtracting corresponding protein values in vehicle treated from the colchicine treated conditions (Ldb3+/– COL minus Ldb3+/– VEH and Ldb3+Alv5/Alv4 COL minus Ldb3+Alv5/Alv4 VEH). Three independent assays were performed for each sample. See Supplementary Data 5 for source data.

RPPA analysis. The RPPA analysis on proteins isolated from frozen vastus muscle of 4- and 8-month-old Ldb3+/– and Ldb3+Alv5/Alv4 mice (n = 5 per group, total 20 mice) was performed by the Functional Proteomics RPPA Core facility at MD Anderson Cancer Center (Houston, TX). Tissue lysate samples were serially diluted two-fold for 5 dilutions (undiluted, 1:2, 1:4, 1:8; 1:16) and arrayed on nitrocellulose-coated slides in an 11 × 11 format to produce sample spots. Sample spots were then probed with 248 antibodies (159 validated for RPPA) against total and phosphorylated proteins by a tyramide-based signal amplification approach and visualized by DAB colorimetric reaction to produce stained slides. Stained slides were scanned on a Huron TissueScope scanner (Huron Digital Pathology, Ontario, Canada) to produce 16-bit tiff images. Sample spots in tiff images were identified and their densities quantified by Array-Pro Analyzer 6.3. Relative protein levels for each sample were determined by interpolating each dilution curve produced from the densities of the 5-dilution sample spots using a “standard curve” (SuperCurve) for each slide (antibody). SuperCurve 1.3.0 (via SuperCurveGUI_2.1.1) was constructed by a script in R-package. All relative protein level data points were normalized for protein loading and transformed to linear values, which were designated “Normalized Linear” or “NormLinear” and were used for expression analysis. Only antibodies with quality control scores above 0.8 in the heatmaps were included in analyses. To quantify differentially expressed proteins between age and gender-matched 4- and 8-month-old Ldb3+/– and Ldb3+Alv5/Alv4 littermates, raw abundances detected per sample were log transformed (base 2), median centered, and variance-stabilized (median absolute deviation) using a R-package. Proteins having an absolute difference of means ≥ 1.5x and a corrected p < 0.05, after a modified t-test under Benjamini–Hochberg false discovery rate multiple comparison correction (rounded to the nearest hundredth) were deemed to be differentially expressed between the groups. See Supplementary Data 6 and 7 for source data.

Statistics and reproducibility. The statistical analyses were performed using either GraphPad Prism 8 or R package. All individual data points are plotted as dot plot or bar-dot plot and presented as mean ± SEM or SD throughout the manuscript. Statistical testing method, sample size, replicates of experiments, and the p value are indicated in figure legends. A two-way ANOVA using the Bonferroni’s multiple comparison test or an unpaired two-tailed t-test were done to determine differences between groups. Statistical significance was defined as p < 0.05. Welch modified t-test under Benjamini–Hochberg false discovery rate multiple comparison correction was applied to identify differentially expressed proteins between genotypes in the RPPA analysis using a R-package. Statistical significance was defined as absolute difference of means ≥ 1.5x and a corrected p < 0.05.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request. Source data underlying plots shown in main figures are provided in Supplementary Data. Full data are shown in Supplementary Fig. 6 in Supplementary Information.
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Acknowledgements
The study was supported by the Intramural Research Program (IRP) of the National Institute of Neurological Disorders and Stroke (NINDS) and National Cancer Institute (NCI). Mouse behavior studies were supported by the National Institute of Mental Health IRP Rodent Behavioral Core (MH002952). The authors thank Eileen Southon, Susan Reid, and Linus Tessarollo (NCI) for knock-in ES cell production and microinjections; Virginia Tanner-Crocker and Dr. Susan Cheng for tissue embedding and sectioning for electron microscopy (NINDS Electron microscopy Core), Dr. Michael Eckhaus for heart histology (Division of Veterinary Resources, Diagnostic and Research Services Branch), Nathan Sarkar (NINDS) and Kory Johnson (NINDS Bioinformatics Section) for bioinformatic support, and Alan Hoofring for designing diagram figures (NIH Medical Arts Design Section). The Functional Proteomics Reverse Phase Protein Array Core Facility at MD Anderson Cancer Center is supported by NCI #CA18672 grant.

Author contributions
A.M. designed the experiments, obtained funding, and supervised the study. P.P., Y.B., K.S., J.M., C.O., R.O., L.M., M.K., J.H., S.S., S.K.S., and A.M. acquired, analyzed, and interpreted the experimental data. P.P., Y.B., K.S., and A.M. prepared the figures. P.P., Y.B., and A.M. wrote the manuscript. P.P and Y.B. contributed equally. All authors approved the content of the manuscript.

Funding
Open Access funding provided by the National Institutes of Health (NIH).

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material at https://doi.org/10.1038/s42003-021-01864-1.

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