Leiomodin-3 dysfunction results in thin filament disorganization and nemaline myopathy

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Nemaline myopathy (NM) is a genetic muscle disorder characterized by muscle dysfunction and electron-dense protein accumulations (nemaline bodies) in myofibers. Pathogenic mutations have been described in 9 genes to date, but the genetic basis remains unknown in many cases. Here, using an approach that combined whole-exome sequencing (WES) and Sanger sequencing, we identified homozygous or compound heterozygous variants in LMOD3 in 21 patients from 14 families with severe, usually lethal, NM. LMOD3 encodes leiomodin-3 (LMOD3), a 65-kDa protein expressed in skeletal and cardiac muscle. LMOD3 was expressed from early stages of muscle differentiation; localized to actin thin filaments, with enrichment near the pointed ends; and had strong actin filament-nucleating activity. Loss of LMOD3 in patient muscle resulted in shortening and disorganization of thin filaments. Knockdown of lmod3 in zebrafish replicated NM-associated functional and pathological phenotypes. Together, these findings indicate that mutations in the gene encoding LMOD3 underlie congenital myopathy and demonstrate that LMOD3 is essential for the organization of sarcomeric thin filaments in skeletal muscle.

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Leiomodin-3 dysfunction results in thin filament disorganization and nemaline myopathy


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Nemaline myopathy (NM) is a genetic muscle disorder characterized by muscle dysfunction and electron-dense protein accumulations (nemaline bodies) in myofibers. Pathogenic mutations have been described in 9 genes to date, but the genetic basis remains unknown in many cases. Here, using an approach that combined whole-exome sequencing (WES) and Sanger sequencing, we identified homozygous or compound heterozygous variants in LMOD3 in 21 patients from 14 families with severe, usually lethal, NM. LMOD3 encodes leiomodin-3 (LMOD3), a 65-kDa protein expressed in skeletal and cardiac muscle. LMOD3 was expressed from early stages of muscle differentiation; localized to actin thin filaments, with enrichment near the pointed ends; and had strong actin filament-nucleating activity. Loss of LMOD3 in patient muscle resulted in shortening and disorganization of thin filaments. Knockdown of lmod3 in zebrafish replicated NM-associated functional and pathological phenotypes. Together, these findings indicate that mutations in the gene encoding LMOD3 underlie congenital myopathy and demonstrate that LMOD3 is essential for the organization of sarcomeric thin filaments in skeletal muscle.

Introduction

Nemaline myopathy (NM) is a common form of congenital myopathy, affecting approximately 1 in 50,000 individuals, and is defined by the presence of nonprogressive generalized muscle weakness and numerous electron-dense protein inclusions (nemaline bodies or rods) in skeletal myofibers (1). The most severely affected
children with NM die in the neonatal period from respiratory insufficiency. Less severely affected patients often have difficulties walking and swallowing and require nocturnal respiratory support. Nine genes have been linked to NM to date; NEB, ACTA1, TPM3, TPM2, CFL2, and TNNT1 all encode components of the sarcomeric thin filament in skeletal muscle, while KBTBD13, KLHL41, and KBTBD13 sarcomeric thin filament in skeletal muscle, while TPM3, CFL2, or TNNT1 are involved in the function of the thin filament.

Whole-exome sequencing (WES) and other approaches that use next-generation sequencing technology have revolutionized gene discovery by allowing rapid, cost-effective screening of large regions of the genome for potentially pathogenic variants. To identify further genetic causes of NM, we used WES on children with NM who lacked a genetic diagnosis after standard investigations. The Journal of Clinical Investigation

**Results**

*Mutations in LMOD3 are a new genetic cause for NM and most mutations result in complete loss of leiomodin-3 protein expression.* WES in 2 unrelated families with NM identified likely mutations in LMOD3, which encodes leiomodin-3 (LMOD3). In the consanguineous families 1, a homozygous frameshift variant in LMOD3 was identified in both affected siblings. In nonconsanguineous family 14, WES identified previously unreported compound heterozygous LMOD3 variants in both affected children. In both families, segregation was consistent with autosomal recessive inheritance.

Genetic screening of the 3 coding exons of LMOD3 by Sanger sequencing, WES, or whole-genome sequencing in over 540 additional genetically unresolved probands with NM identified likely pathogenic variants in 17 patients from 12 additional families (Figures 1 and Table 1). Segregation studies were consistent with autosomal recessive inheritance. Most other common genetic causes of NM had been excluded previously in patients in whom LMOD3 variants were identified (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI75199DS1). LMOD3 variants were distributed throughout the gene and most population and is very unlikely to be associated with severe NM in families 8 and 10 by chance alone. Amino acid G326 is conserved in the LMOD1, LMOD2, and LMOD3 and TMOD1, TMOD2, and TMOD3 proteins and is located within the leucine-rich repeat domain of LMOD3 (Figure 1), which is predicted to bind actin. In silico prediction of the functional consequences of this variant was supportive of pathogenicity (PolyPhen-2 score 1.00 = probably damaging, SIFT score 0 = damaging). Together, these data support the hypothesis that the c.976G>C (p.G326R) variant disrupts gene function and is pathogenic.

Although tissue was not available from affected individuals in all families, Western blotting confirmed that many mutations result in no detectable LMOD3 protein in muscle, with the exception of patient 14a, in whom protein expression from both mutant alleles was demonstrated (Figure 2A and Table 1). To confirm these results, we analyzed primary myoblast cell lines and/or fibroblast cell lines (transformed into myogenic cells using MyoD transduction; refs. 4, 5) derived from the probands of families 12 and 14. Western blot analysis confirmed the absence of LMOD3 expression in myotubes from family 12 and the expression of both mutant forms of LMOD3 in myotubes from patient 14a (Figure 2B). In addition, we confirmed that the polyclonal LMOD3 Ab we used was able to detect N-terminal protein fragments 51–amino acids long (Supplemental Figure 1), corresponding to the shortest predicted LMOD3 truncation in our cohort.

NM patients with LMOD3 mutations typically have severe congenital NM and distinctive nemaline body morphology on electron microscopy. NM patients with LMOD3 mutations (LMOD3-NM) presented with severe congenital NM in 90% of cases (13 of 14 families) (Table 2). Antenatal manifestations included polyhydramnios (62% of patients), decreased or absent fetal movements (48% of patients), and joint contractures (often multiple, 48% of patients). Thirty-five percent of patients were born prematurely. All patients had severe generalized hypotonia and weakness at birth, respiratory insufficiency, feeding difficulties, and bulbar weakness (Figure 3, A and B, and Table 2). Twenty-nine percent of patients had ophthalmoplegia, which is uncommon in other genetic forms of NM. Most patients died in the neonatal period from respiratory failure. No cardiac abnormalities were reported. Two sisters from...
family 14 are the only known surviving patients, currently aged 10 and 4 years. Lower limb muscle MRI in patient 14a demonstrated widespread atrophy and fatty infiltration of thigh and lower leg muscles, with relative sparing of some muscles, including vastus lateralis, gracilis, semimembranosus, semitendinosus, and extensor digitorum longus (Supplemental Figure 2).

Light microscopy images of skeletal muscle biopsies were available from 11 patients, and electron microscopy (EM) images were available from 6 patients. Histological assessment revealed nemaline bodies and atrophic myofibers in all LaMOD3-NM muscle biopsies. Interstitial connective tissue was increased in all biopsies and had almost completely replaced myofibers in 5 of 11 biopsies (Figure 3D). On EM images, many nemaline bodies resembled short thickened Z-discs (seen in all 6 patients for whom EM images were available; Figure 3, G and H), often in doublets interconnected by filaments (present in 4 of 6 patient biopsies; Figure 3D). Some nemaline bodies were surrounded by a short thin filament “fringe” 60–220 nm in length (Figure 3, I and J; present in 3 of 6 patient biopsies).

were normalized to cross-sectional area to confirm that weakness of lmod3 MO was not solely due to reduced lmod3 MO muscle size. LMOD3 is expressed in skeletal muscle throughout life and localizes to the actin thin filament. The leiomodins are members of the tropomodulin protein family, and 3 leiomodin genes have been identified so far: LMOD1, LMOD2, and LMOD3. LMOD1 is expressed predominantly in smooth muscle, while LMOD2 and LMOD3 are mainly found in skeletal and cardiac muscle (6, 7).

We performed protein studies in healthy muscle to characterize LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. Western blot analysis on control human heart and skeletal muscle biopsies demonstrated LMOD3 expression and localization. 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Localization of LMOD3 within skeletal muscle sarcomeres was assessed by immunolabeling of stretched adult human control muscle and differentiated cultured control myotubes. LMOD3 was highly expressed in nonstriated areas of developing myotubes and showed a granular cytoplasmic staining (Figure 5, D and E, square 2). In areas in which myosin and actin filaments were organized into sarcomeres, LMOD3 showed intense staining of the M band region, which colocalizes with thin filament pointed ends in unstretched sarcomeres. Less intense staining was observed along thin filaments, and the Z-discs were unstained (Figure 5, D and E, square 1). Expression of mCherry-labeled LMOD3 in cultured quail myotubes provides additional evidence for localization to the M line/thin filament pointed end region (Supplemental Figure 4).

In stretched mature human skeletal muscle, we observed 3 staining patterns: (a) exclusive staining near the pointed end of thin filaments, (b) combined staining near the pointed end and along the thin filament, and (c) predominant staining along the thin filament (Supplemental Figure 5). In 4 of 5 biopsies, we observed more than one staining pattern in different myofibers, and no correlation with fiber type (determined by myosin heavy chain [MHC] analysis) or age was found (Supplemental Figure 6). We compared the breadth of LMOD3 staining across a full sarcomere to the thin filament length, measured using phalloidin in the same sarcomere, and found that, while they closely correlated in fast fibers, the breadth was significantly shorter in slow fibers ($P < 0.0001$, paired t test). This suggests that LMOD3 binding may extend to the thin filament cap in fast fibers but ends before the cap in slow fibers (Figure 6, E and F). When the LMOD3 signal was enriched near the pointed end, the band appeared wider than those obtained for TMOD1 and TMOD4. At higher magnification, the wide LMOD3 band could occasionally be resolved into 2 narrow bands (Figure 6D and refs. 8, 9). The intense LMOD3 stained peak near the pointed end was further away from the Z-disc in fast myofibers (1.07 ± 0.13 μm, $n = 14$) compared with slow myofibers (0.91 ± 0.14 μm, $n = 15$, $P = 0.0044$, t test; Figure 6E). Notably, the peak of LMOD3 staining in both myofiber types was significantly closer to the Z-disc than that of TMOD1 (average of fast and slow myofibers), which was located 1.26 ± 0.16 μm ($n = 4$) from the Z-disc, suggesting that LMOD3 may be located near, but not at, the thin filament pointed end.
Table 2. Clinical features of LMOD3-NM

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Genotype</th>
<th>Ethnicity</th>
<th>Sex</th>
<th>Clinical subtype*</th>
<th>Clinical features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>p.S47fs*13</td>
<td>Algerian</td>
<td>F</td>
<td>SC</td>
<td>Deceased (neonatal period)*. Polyhydramnios, preterm delivery (30/40), arthrogryposis, fractures (bilateral femoral).</td>
</tr>
<tr>
<td>3b</td>
<td>p.M52*</td>
<td>Portuguese</td>
<td>F</td>
<td>SC</td>
<td>Alive at 1 month, lost to follow-up. Polyhydramnios, contractures.</td>
</tr>
<tr>
<td>4</td>
<td>p.W77*</td>
<td>Japanese</td>
<td>M</td>
<td>SC</td>
<td>Alive at 4 months, lost to follow-up.</td>
</tr>
<tr>
<td>9</td>
<td>p.F287Sfs*3</td>
<td>Italian</td>
<td>F</td>
<td>SC</td>
<td>Deceased (4 months). Polyhydramnios, decreased fetal movements, preterm delivery (34/40), opthalmoplegia, contractures.</td>
</tr>
</tbody>
</table>

*Classification of clinical subtype of NM relates to the European Neuromuscular Centre classification of NM (1). *Neonatal period indicates less than 28 days of age. *Corresponds to BDS-1120. TC, typical congenital NM; SC, severe congenital NM; F, female; M, male; 30/40, gestational age of 30 weeks.

LMOD3 is a potent actin nucleator and has a weaker binding affinity to tropomyosin compared with that of LMOD2. Previous studies have shown that LMOD2 localizes to the pointed end of actin filaments in cardiomyocytes and is a potent actin filament nucleator (10–12). We used a pyrene actin nucleation assay to compare the actin nucleation properties of LMOD2 and LMOD3 and observed that both leiomodins led to a marked increase in rate of actin polymerization, with an equivalent dose dependence (Figure 7A).

The leiomodins share a single tropomyosin (TM) binding domain with TMOD, and amino acid sequence analysis showed that LMOD3 is more divergent from TMOD1 than LMOD2 in this region (Figure 1 and Supplemental Figure 7, amino acids 29–40 in LMOD3). We performed far-UV circular dichroism (CD) spectral studies to test binding of an N-terminal α-TM peptide to LMOD2 and LMOD3 peptides. CD spectral studies indicated complex formation for TM/LMOD2 and TM/LMOD3 peptide mixtures (Figure 7C). The increase in α-helicity was stronger for LMOD2, indicating a higher TM binding affinity. Unfolding curves of TM/LMOD peptide complexes indicated that the LMOD2 peptide binds approximately 4-fold more strongly to the α-TM N-terminal peptide than the LMOD3 peptide ($K_{d} = 3.5 \pm 0.5 \mu M$ for TM/LMOD3 and $0.9 \pm 0.3 \mu M$ for TM/LMOD2, $n = 3$; Figure 7B).

Thin filament structure is altered in patients with LMOD3-NM and lmod3 KO zebrafish. We assessed thin filament architecture in LMOD3-NM by dissecting longitudinal myofiber bundles from 9 patient biopsies and staining stretched bundles with phalloidin, which labels filamentous actin (Figure 8, A–J). In half of the biopsies we found no discernible sarcromeric structure (patients 4, 5, 6, and 9). In all other patient biopsies we observed varying degrees of thin filament shortening and disorganization (patients 1a, 7, 12a, 14a; Supplemental Table 2). Four biopsies from three patients (patients 1a, 12a, and 14a) also contained regions with well-organized sarcromeres, and such regions were also seen on EM images (Figure 3, G and H).
We were able to perform force measurements at SLs of 2.0 to 3.5 μm in the 3 biopsies from patients 14a and 12a, the biopsies with the best sarcomeric organization (Figure 8, K–M). Highly variable contractile force (patient 1a) or no measurable force (patients 4 and 7) was obtained in other biopsies, likely because muscle structure was too disordered. Interestingly, maximal force normalized to cross-sectional area in both biopsies from patient 14a was the same as that in controls (mean force: 62.06 ± 15.82 and 61.25 ± 19.56 mN/mm² in patient myofibers and 61.94 ± 22.07 mN/mm² in 5 controls), while patient 12a myofibers produced 57% less force than controls (26.72 ± 6.34 mN/mm²; P < 0.0001) (Figure 8K). In all 3 biopsies, there was a leftward shift in the force/SL curve, consistent with decreased thin filament lengths in these patients (Figure 8I). Patient 12a produced less specific force at all SLs.

Several thin filament pointed end proteins are dysregulated in LMOD3-NM. We investigated the expression of LMOD2, another striated muscle leiomodulin, and the 2 sarcomeric skeletal muscle tropomodulins, TMOD1 and TMOD4, in available patient muscle biopsies using Western blot analysis. We found increased LMOD2 expression in all patients tested, compared with age-matched control biopsies. TMOD1 expression was variable in both groups, and TMOD4 expression was uniformly decreased in patient biopsies (Figure 9). TMOD1 and TMOD4 immunostaining on stretched patient bundles showed that both proteins still localized to the pointed end of thin filaments in myofibrils with discernible sarcomeric structure (Supplemental Figures 8 and 9). However, in the fiber bundles extracted from muscle from patient 12a, TMOD1 labeling was enriched in some areas containing protein accumulations (Supplemental Figure 8). LMOD2 and LMOD3 staining was not consistently detected in stretched bundles from either controls or patients, suggesting that the process of preparing stretched myofiber bundles from frozen biopsies disrupts staining with these Abs, unfortunately.

Discussion
We describe 21 patients from 14 families who have compound heterozygous or homozygous mutations in LMOD3 consistent with...
autosomal recessive inheritance, associated with severe NM, establishing LMOD3 as a new cause of this disorder. As further confirmation, we developed a zebrafish model of LMOD3-NM and demonstrated abnormalities in skeletal muscle organization and function after LMOD3 knockdown.

The clinical presentation of LMOD3-NM is characterized by early-onset severe generalized muscle weakness and hypotonia, with respiratory insufficiency and feeding difficulties. The severe congenital subtype of NM is relatively uncommon, accounting for only 16% of patients with NM in a large case series (14). Ninety percent of patients with LMOD3-NM identified in the current study had severe congenital NM, establishing LMOD3 as an important cause of this phenotype, in addition to ACTA1, NEB, KLHL40, and KLHL41. Features commonly seen in LMOD3-NM, which may serve as clinical clues for the disorder, include arthrogryposis or congenital contractures, ophthalmoplegia, and a history of prematurity, reduced fetal movements, and polyhydramnios. We also report a distinctive ultrastructural appearance of nemaline bodies in LMOD3-NM. Many of the nemaline bodies resembled thickened Z-disc remnants, which often appeared in pairs, surrounded

Figure 4. Morpholino knockdown of lmod3 in zebrafish recapitulates features of LMOD3-NM. (A) Representative bright-field (top) and birefringence (bottom) images of control and lmod3 knockdown zebrafish morphants 3 days after fertilization (dpf). Note the shorter body size, bent tail, and reduced birefringence of lmod3 MO. (B) RT-PCR from embryos injected with control morpholinos (lanes 2, 3, 6, and 7) or lmod3 morpholinos (lanes 4 and 8). Knockdown of the lmod3 gene was achieved using 2 morpholinos directed against different splice sites. PCR, performed with primers targeting lmod3 spanning exons 1–3 (lanes 2–4) and exons 2–4 (lanes 6–8), demonstrates successful interruption of splicing by the lmod3 morpholinos. LMOD3 protein expression was markedly reduced in lmod3 MO embryos by (B) Western blotting and (C) immunohistochemistry. (D) Isolated myofibers from lmod3 MO immunostained with α-actinin show protein accumulations resembling nemaline bodies (white arrows). No accumulations were observed in control MO (data not shown). (E) Spontaneous embryo coiling 24 hours after fertilization (hpf) was significantly reduced in lmod3 MO. (F) A significant reduction in touch-evoked escape response (0–3 response scale) was observed 48 hours after fertilization in lmod3 MO. (G) Contractile force generated by lmod3 MO trunk muscle (3 days after fertilization) was significantly reduced. All error bars represent mean ± SEM. Scale bar: 5 μm (C and D). ****P < 0.0001, 2-tailed paired t test. CSA, cross-sectional area.
and p.R401* mutant LMOD3 alleles in skeletal muscle. Patients 14a and 14b are the only 2 patients known to have survived beyond early childhood, which suggests that one of these mutant proteins (or both) retains some function, conferring a milder phenotype. The fact that loss of LMOD3 leads to severe muscle dysfunction indicates that LMOD3 plays an essential role in skeletal muscle, which has not been recognized previously.

A striking feature in muscles from many patients with LMOD3-NM is replacement of myofibers with connective tissue. After staining of actin filaments in individual myofibers with phalloidin, no discernible sarcomeres were seen in half of the patient muscle biopsies, indicating widespread disorganization of muscle ultrastructure. This appearance is rare in other forms of NM and may assist in diagnosing this condition. Interestingly, even though we demonstrated that LMOD3 is present in cardiac muscle, cardiac involvement has not been reported so far in LMOD3-NM, and it is possible that LMOD2 may compensate for loss of LMOD3 in cardiac muscle.

Most LMOD3 mutations we identified are nonsense or frameshift mutations that would truncate the LMOD3 protein if expressed. In almost all patients with LMOD3-NM for whom muscle biopsies were available, mutations in LMOD3 were associated with a loss of LMOD3 expression. The only exception was family 14, in which we found expression of both the p.N367del and p.R401* mutant LMOD3 alleles in skeletal muscle. Patients 14a and 14b are the only 2 patients known to have survived beyond early childhood, which suggests that one of these mutant proteins (or both) retains some function, conferring a milder phenotype. The fact that loss of LMOD3 leads to severe muscle dysfunction indicates that LMOD3 plays an essential role in skeletal muscle, which has not been recognized previously.

A striking feature in muscles from many patients with LMOD3-NM is replacement of myofibers with connective tissue. After staining of actin filaments in individual myofibers with phalloidin, no discernible sarcomeres were seen in half of the patient muscle biopsies, indicating widespread disorganization of muscle ultrastructure.
structure. A paucity of contractile units is likely the main cause of muscle weakness in most patients. When sarcomeres were present, a range of structural abnormalities were seen, ranging from thickened Z-disc–like structures without thin filaments, to Z-discs with shortened thin filaments, to sarcomeres with near-normal structure. In addition, lmod3 MO zebrafish lacked clearly defined pale zones immediately adjacent to M lines. This appearance could be caused by abnormal myosin filament/M line structure or due to loss of distinct H bands, a finding reported in Neb knockout mice (15). The H zone represents the region of the sarcomere that only contains thick filaments, with the H zone boundary created by the well-aligned thin filament pointed ends at either side. In Neb knockout mice, variability in thin filament lengths leads to loss of a clear boundary for the H zone (15). To obtain further information about whether variable thin filament lengths may be a common finding in LMOD3-NM, we analyzed the force/SL relation—ship in myofibers dissected from patient biopsies. The leftward shift in the force/SL curves in all biopsies tested confirms that, even in myofibers with well-ordered sarcomeres, populations of shortened thin filaments are present and likely contribute to muscle weakness, a mechanism of disease previously linked to other forms of NM (16). Dysregulation of the tropomodulins, particularly TMOD4, in skeletal muscle, may also contribute to muscle weakness in LMOD3-NM.
The N-termini of LMOD1, LMOD2, and LMOD3 have approximately 40% sequence identity with the TMODs, sharing 2 actin-binding domains and 1 of 2 TM-binding sites (10). In addition, the C-termini of the leiomodins contain a third actin-binding domain, which is not present in the TMODs but which is a characteristic of actin filament-nucleating proteins (Figure 1 and ref. 10). Previous studies have shown that LMOD2 localizes near the pointed end of thin filaments in cardiomyocytes and functions as a potent actin filament nucleator in vitro (10–12).

To investigate the biological functions of LMOD3, we performed a range of studies in control muscle samples and using recombinant protein. Tsukada et al. showed that LMOD2 regulates thin filament lengths in cardiomyocytes likely by competing with TMOD, which caps actin filaments, and by promoting actin polymer extension at the pointed end of thin filaments (12). We hypothesized that LMOD3 may play a similar role in skeletal muscle. Our studies using recombinant proteins and stretched quadriceps muscle show that, like all other members of the TMOD family, LMOD3 binds both actin and TM and localizes close to the pointed ends of thin filaments, a key site for actin turnover and control of thin filament length in skeletal muscle. We also demonstrated that LMOD3, like LMOD2, is an effective catalyst of actin nucleation. In addition, loss or dysfunction of LMOD3, which defines LMOD3-NM, was associated with short thin filaments in immunofluorescent and force/SL measurement studies in patient myofibers. These data are all consistent with a central role for LMOD3 in modulating thin filament length in skeletal muscle.

In LMOD3-NM patient muscle, we observed an upregulation of LMOD2, another striated muscle leiomodin that is expressed at higher levels in cardiac muscle compared with skeletal muscle. This may represent a specific upregulation of a homologous protein as a compensatory mechanism, a process that is well described to occur in patients with autosomal recessive mutations in ACTA1, which encodes α-skeletal actin, in whom cardiac actin is markedly upregulated in skeletal muscles after birth (17, 18). However, the presence of such severe muscle weakness in patients with LMOD3-NM suggests that the LMOD2 upregulation that occurs compensates poorly for the loss of functional LMOD3. The fact...
may have several functions; it may bind near the pointed end of thin filaments to modulate filament length and also bind along the length of the thin filament to perhaps function as a thin filament stabilizer. Further studies are required to confirm this hypothesis.

In a recently published study, LMOD3 and nebulin were identified as major binding partners of KLHL40, loss of which results in a severe lethal form of NM associated with destabilization of thin filament proteins (19, 20). KLHL40 was found to promote stability of LMOD3 by blocking its ubiquitination, and loss of KLHL40 was associated with almost complete absence of LMOD3 protein in skeletal muscles of mice and KLHL40-deficient patients. These data support a pivotal role for LMOD3 in the stability of the sarcomere thin filament. The newly identified interaction among LMOD3, nebulin, and KLHL40 provides the “missing link” between thin filament proteins and the kelch (BBK) protein family and suggests a common pathway for the pathogenesis of a number of different genetic forms of NM.

In conclusion, we show that autosomal recessive mutations in LMOD3 are a new and important cause of severe congenital NM and that LMOD3 plays a critical role in skeletal muscle biology. Multiple lines of evidence point to a role for LMOD3 as a key regulator of thin filament length in skeletal muscle. This study emphasizes the power of WES in identifying the genetic basis of NM.
This reduced the candidate regions to 3% of the genome. WES was then performed in the index case. WES was performed in a similar manner to that described for family 14. Exome libraries were captured using the Agilent SureSelect V3. SNPs and small indels were called using SAM tools and annotated using ANNOVAR. Homozygous single nucleotide variants mapping to candidate loci were filtered as described for family 14.

Genetic screening for additional LMOD3 mutations
Bidirectional Sanger sequencing of LMOD3 was performed on biobanked DNA in 476 additional genetically unresolved patients with NM in Helsinki, London, Perth, Sydney, and Tokyo. LMOD3 was PCR amplified from genomic DNA in 4 fragments covering 3 exons using standard methods (PCR primer sequences in Supplemental Table 3). An additional 68 cases were screened by WES or whole-genome sequencing in Boston through the Boston Children’s Hospital Gene Partnership Program. WES in these cases was performed as previously described (3). In cases screened by whole-genome sequencing,

Figure 9. Levels of LMOD2 and TMOD4 are altered in LMOD3-NM. (A) Western blot analysis of thin filament pointed end proteins LMOD2, TMOD1, and TMOD4 in patients with LMOD3-NM and age-matched controls. Coomassie-stained MHC, sarcomeric actin, GAPDH, and α-actinin-2 served as loading controls. (B) Histograms show normalized protein levels for TMOD1, TMOD4, and LMOD2 in patients with LMOD3-NM and controls. TMOD1 expression levels are variable in both patients and controls, with no consistent difference between these groups. TMOD4 levels are consistently reduced in patients with LMOD3-NM compared with age-matched controls. LMOD2 expression is upregulated in patients. Densitometry was performed using ImageJ, and values were normalized to α-actinin-2 levels and are scaled to mean control level = 1.0. Patients with low or no expression of muscle proteins were excluded from analysis (e.g., patient 4). Dotted lines show the average results for patients and controls, respectively.
this was performed by Complete Genomics and processed through their Standard Pipeline v. 2.2.

Abs
A polyclonal rabbit anti-LMOD3 Ab was purchased from Proteintech (14948-1-AP) and used at a dilution of 1:10,000 for Western blot and 1:350 for immunohistochemistry. Two polyclonal rabbit LMOD2 Abs (Abcam, 108022, and Santa Cruz, sc-135493, S-12) were used for Western blot at dilutions of 1:250 and 1:500, respectively. The specificity of LMOD2 and LMOD3 Abs was confirmed using recombinant proteins expressed in bacteria and COS cells (see Supplemental Figure 1). The rabbit α-actinin-2 Ab (clone 4A3, 1:250,000 for Western blot) was developed in-house by A.H. Beggs. Mouse α-actinin Ab was purchased from Sigma-Aldrich (EA-53, 1:400 immunohistochemistry). Total sarcomeric actin levels were assessed using a mouse sarcomeric actin Ab from Sigma-Aldrich (SC5, 1:10,000 for Western blot). The GAPDH Ab was obtained from Millipore (MAB374, 1:10,000 for Western blot). Developmental MHC Ab, which recognizes the embryonic MHC isoform, was purchased from Novocastra Laboratories Ltd (NCL-MHCd, dilution 1:200 for Western blot), cardiac actin Ab was purchased from American Research Product Inc. (1:500 for Western blot), slow MHC Ab was purchased from Chemicon International Inc. (MAB1628, dilution 1:3,000 Western blot, 1:1,000 immunohistochemistry), and fast MHC Ab was purchased from Sigma-Aldrich (MY-32, 1:800 immunohistochemistry). TMOD1 and TMOD4 Abs were produced in-house by V.M. Fowler (dilution for immunohistochemistry was 1:50 and 1:100 for TMOD4 and TMOD1, respectively; ref. 22). Secondary Abs (Alexa Fluor 555 rabbit and Alexa Fluor 647 mouse, 1:200) for immunohistochemistry were obtained from Molecular Probes, Invitrogen. HRP-conjugated secondary Abs for Western blot were purchased from GE Healthcare (1:2,000).

Western blot
Western blotting methods for human samples and cells were based on methods previously described with modifications (23). Protein concentration was determined via bicinchoninic acid BCA assay per manufacturer’s instructions (Pierce, Thermo Fisher Scientific). Five μg total muscle protein was separated on precast 10% SDS-PAGE gels (NuPAGE Novex Bis-Tris Gels, Invitrogen, Life Technologies, or Bio-Rad) for the detection of LMOD3, followed by transfer of proteins onto nitrocellulose membrane for 1 hour in Tris-Glycine buffer containing 0.075% SDS. Membranes were blocked in 5% skim milk (diluted in PBS 0.1% Tween 20) for 1 hour, followed by incubation in primary Ab for 2 hours at room temperature or for 16 hours at 4°C. Unbound primary Ab was removed by washing for 25 minutes in PBS 0.1% Tween 20, followed by 15 minutes of blocking. Secondary HRP Ab (1:2,000 in block for 1 hour at room temperature) and ECL Chemiluminescence Detection System (GE Healthcare Life Science) were used for detection. In some cases membranes were stripped of bound Ab by washing in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 0.1 M β-mercaptoethanol for 30 minutes. Stripping buffer was removed by washing 5 times for 1 minute in large volumes of PBS 0.1% Tween 20, and membranes were reprobed. Membranes were stained for total proteins with Coomassie Blue Brilliant (Sigma-Aldrich).

For Western blot studies of zebrafish samples, protein extracts were established from flash frozen fish embryo samples (3 days after fertilization) using a RIPA protein extraction buffer with complete Mini Protease inhibitor (Roche) and a Dounce tissue homogenizer (Pierce Biotechnology). Approximately 60 μg protein was loaded per sample, resolved by polyacrylamide gel electrophoresis on 10% gels, and transferred to polyvinylidene fluoride membrane. After blocking in PBS containing 0.1% Tween, 5% dried milk, and 3% BSA, the membrane was probed with LMOD3 Ab (1:5,000, Proteintech) and TMOD1 and TMOD4 (1:1,000, see Abs), with an actin Ab (1:10,000, Sigma-Aldrich) as loading control. Secondary Abs were used at 1:5,000 (Bio-Rad), and blots were developed using electrochemiluminescence reagent (GE Biosciences).

Immunohistochemistry to determine LMOD3 localization in control muscle samples
Control quadriceps muscle tissue from 5 healthy individuals was fixed in 3% paraformaldehyde in a stretched position, embedded in TissueTek, and frozen in 2-methylbutane cooled in liquid nitrogen. Longitudinal 8-μm-thick cryosections were collected on glass slides and blocked for 1 hour in 4% BSA prepared in PBS, followed by incubation in primary Abs diluted in blocking solution for 2 hours at room temperature. Sections were labeled with rabbit LMOD3 Ab and slow MHC, fast MHC, or α-actinin-2 Ab. Unbound Abs were removed by washing 4 times in PBS, followed by 15 minutes of blocking as above. Secondary anti-mouse Alexa Fluor 647, anti-rabbit Alexa Fluor 488, and Alexa Fluor 488-conjugated phalloidin (diluted 1:20, Life technology) were incubated on muscle sections for 1 hour. Finally, slides were washed as above and mounted using Immu-Mount (Thermo Scientific). Staining was imaged by confocal microscopy. Five areas were imaged per control biopsy, ensuring sarcomeres were adequately stretched, as determined by phalloidin staining. In each area imaged, the staining pattern was recorded (pointed end staining, staining along the thin filament, and combined pointed end and thin filament staining) and the fiber type was determined with MHC staining.

A subset of images showing high-quality pointed end LMOD3 staining were used to measure the distance from the Z-disc of the LMOD3 peak of intense staining. Measurements were first performed manually using ImageJ (profile blot function). Measurements were confirmed using Distributed Deconvolution Analysis (24).

Tissue culture
Primary patient and control fibroblast cultures were transduced with a MyoD-lentiviral vector to induce myogenic conversion as described previously (4). Primary patient and control myoblasts and MyoD-converted fibroblasts were grown to confluence and differentiated in 5% horse serum with 1x ITS for 5 to 8 days to induce myotube formation. Western blot was performed using 5 μg cell lysate from differentiated cells.

Imod3 MO zebrafish
Knockdown of imod3 gene expression in zebrafish was achieved by coinjection of morpholinos directed against the splice acceptor site of exon 2 and the splice donor site of exon 3. Morpholinos were injected at a concentration of 0.3 mM. Morpholino experiments were controlled by injecting an identical concentration and amount of standard control morpholino (GeneTools Inc.). All injections were done into 1-cell stage AB zebrafish embryos. All embryos were manually dechorionated at day 1 after fertilization. Histopathology, including immunofluorescence analysis of isolated myofibers and EM of muscle from whole embryos, was performed as previously described (25, 26).
Assessment of muscle function. Spontaneous coiling was assessed in zebrafish 24 hours after fertilization by counting the number of coiling events in a 15-second epoch. Groups of 20 live embryos were videoed together using a Nikon Microscope, and then coiling was measured via examination of video data.

Touch-evoked escape response data was generated as previously described (27).

Contractile properties of whole zebrafish trunk muscles were measured at 3 days after fertilization as previously described (25, 28). The width of the trunk, as viewed from ventral and lateral aspects, was measured, and cross-sectional area was estimated from these measurements assuming an ellipse (28). Values are shown as mean ± SD.

Actin nucleation/polymerization assay

Expression of recombinant leiomodin proteins. Full-length LMOD2 and LMOD3 were expressed and purified as previously described (10). Following purification, proteins were exchanged into 20 mM Tris-HCl, pH 7.4, for biochemical assays.

Actin purification. Chicken pectoral skeletal muscle G-actin was purified from acetone powder as previously described (29) and was kept on ice in 2 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.2 mM ATP, 0.2 mM CaCl₂, 0.01% NaN₃ to use fresh in fluorescence experiments. Actin concentration was determined by measuring difference spectra in 6 M guanidine-HCl between pH 12.5 and 7.0, using extinction coefficients of 2,357 per tyrosine and 830 per tryptophan as previously described (30). G-actin labeled with pyrene-iodoacetamide was prepared as previously described (31). Labeling ratios (~90%) were calculated as previously described (32), and pyrene actin was stored in liquid nitrogen. Before experiments, pyrene actin was defrosted at 37°C and centrifuged at 386,000 g (TLA-100, Beckman) for 30 minutes at 4°C to remove aggregates. The same solution of G-actin was used for all experiments to decrease the measurement error.

Fluorescence measurements. Actin polymerization was monitored by the increase in fluorescence using a Photon Technology International spectrophotometer, Model QM-40 (QuantaMaster-40) (excitation 366 nm and emission 387 nm, with a 2-nm slit). To measure actin nucleation by LMOD, 1.1 μM G-actin (10% pyrenyl-actin) was mixed with 2, 5, 20, 50, and 100 nM LMOD, and the reaction was initiated by addition of 20x polymerization buffer to final concentrations of 10 mM imidazole, pH 7.0, containing 100 mM KCl, 2 mM MgCl₂, and 1 mM EGTA. Spontaneous actin nucleation in the absence of LMOD was used as a control.

TM binding

Peptides. Peptides for CD measurements were synthesized by the Tufts University Core Facility. The TM peptide is a designed acetylated chimeric peptide that consists of 14 N-terminal residues of long α-TM encoded by exon 1a, which contains the TMOD-binding site, and the 18 C-terminal residues of the GCN4 leucine zipper domain, which help to stabilize the coiled-coil structure (33, 34). Note that the corresponding N-terminal sequence of long muscle β-TM encoded by exon 1a is identical to the α-TM sequence. Leiomodin fragments (amino acids 5–42 for mouse LMOD2 and amino acids 6–44 for mouse LMOD3) were designed based on alignment with the first TM-binding site of TMOD1. The synthetic peptides were purified and analyzed on HPLC, and their quality was confirmed using mass spectrometry. Concentrations of peptides were determined by a BCA Protein Assay Kit (Pierce) and by measuring their difference spectra in 6 M guanidine-HCl as previously described (30).

CD measurements. CD measurements were done using an Aviv model 420SF spectrometer in 1-mm cuvettes. The peptide concentrations were 10 μM in 100 mM NaCl, 10 mM Na-Phosphate buffer, pH 7.0. Change of helical content during temperature denaturation was monitored at 222 nm. The dissociation constants, K_D, of the LMOD/TM peptide complexes were determined as previously described (35). Dissociation constants were statistically compared using a 2-tailed t test, and values are shown as mean ± SD.

Single-fiber mechanics and staining of stretched myofiber bundles

Contractile properties were measured in myofiber bundles isolated from frozen muscle biopsies using methods described previously (16, 36). Force response to saturating Ca²⁺ concentrations (pCa 4.5) was measured at different SLs 2.0, 2.2, 2.5, 2.8, 3.2, and 3.5 μm. Forces were normalized to cross-sectional area measured at 2.5 μm SL. For force/SL curves, forces at various SLs were normalized to the highest force obtained. At least 5 bundles were measured per biopsy. MHC isoform content of bundles was determined by SDS-PAGE electrophoresis as described previously (16). Force/SL curves were statistically compared with pooled control values using repeated-measures ANOVA. Values are shown as mean ± SD.

For confocal imaging of phalloidin studies performed on myofiber bundles dissected from frozen LMOD3-NM patient biopsies and age-matched controls, small aluminum clips were glued to a glass slide. Fiber bundles from frozen biopsies were stretched and fixed between the aluminum clips in relaxing buffer with protease inhibitors. Stretched bundles were then fixed using 3% paraformaldehyde in PBS for 20 minutes at 4°C, followed by washes in PBS (5 buffer changes, last wash overnight at 4°C). Bundles were blocked for 1 hour in 1% BSA in PBS and labeled with 1 unit phalloidin conjugated to Alexa Fluor 488 for 48 hours. Unbound phalloidin was rinsed off, and bundles were mounted in Vectashield (Vector Laboratories) with #0 coverslips. Staining was analyzed by confocal microscopy, and thin filament length was measured using ImageJ (version 1.44, from Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/index.html).

Statistics

Statistical analysis was performed as specified in figure legends and in the appropriate methods section. A P value of less than 0.05 was considered significant.

Study approval

Ethical approval for this research was obtained from the Human Research Ethics Committees of the Children’s Hospital at Westmead (10/CHW/45), the University of Western Australia, Bicêtre University Hospital (N100054), the Children’s Hospital of the University of Helsinki, the Japanese National Center of Neurology and Psychiatry, and the Boston Children’s Hospital Institutional Review Board (03-08-128R). Written informed consent was obtained for genetic testing of participants, biobanking of patient muscle and DNA, and publishing of patient photographs. Mouse muscle analyses were performed using an Animal Care and Ethics Committee protocol locally approved at the Children’s Hospital at Westmead. Zebrafish were housed and bred under the University of Michigan’s University Com-
mittee on the Use and Care of Animal–approved conditions, and all experiments were done via a protocol approved by the ethics board of the University of Michigan.

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