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Mutation-Specific Effects on Thin Filament Length in Thin Filament Myopathy

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Abstract

Objective—Thin filament myopathies are among the most common nondystrophic congenital muscular disorders, and are caused by mutations in genes encoding proteins that are associated with the skeletal muscle thin filament. Mechanisms underlying muscle weakness are poorly

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Potential Conflicts of Interest

Nothing to report.

Additional supporting information can be found in the online version of this article.

understood, but might involve the length of the thin filament, an important determinant of force generation.

Methods—We investigated the sarcomere length-dependence of force, a functional assay that provides insights into the contractile strength of muscle fibers as well as the length of the thin filaments, in muscle fibers from 51 patients with thin filament myopathy caused by mutations in *NEB*, *ACTA1*, *TPM2*, *TPM3*, *TNNT1*, *KBTBD13*, *KLHL40*, and *KLHL41*.

Results—Lower force generation was observed in muscle fibers from patients of all genotypes. In a subset of patients who harbor mutations in *NEB* and *ACTA1*, the lower force was associated with downward shifted force–sarcomere length relations, indicative of shorter thin filaments. Confocal microscopy confirmed shorter thin filaments in muscle fibers of these patients. A conditional *Neb* knockout mouse model, which recapitulates thin filament myopathy, revealed a compensatory mechanism; the lower force generation that was associated with shorter thin filaments was compensated for by increasing the number of sarcomeres in series. This allowed muscle fibers to operate at a shorter sarcomere length and maintain optimal thin–thick filament overlap.

Interpretation—These findings might provide a novel direction for the development of therapeutic strategies for thin filament myopathy patients with shortened thin filament lengths.

Nemaline myopathy and congenital fiber type disproportion are among the most common nondystrophic congenital muscular disorders.¹ Genes that are implicated in these myopathies encode proteins that are either components of the skeletal muscle thin filament, including nebulin (*NEB*), skeletal muscle alpha-actin1 (*ACTA1*), beta-tropomyosin 2 (*TPM2*), alpha-tropomyosin 3 (*TPM3*), troponin T type 1 (*TNNT1*), cofilin-2 (*CFL2*), and leiomodin-3 (*LMOD3*), or are thought to contribute to stability or turnover of thin filament proteins, such as kelch repeat and BTB (POZ) domain containing 13 (*KBTBD13*), and kelchlike family members 40 (*KLHL40*) and 41 (*KLHL41*).^{2–6} Hence, muscle diseases caused by mutations in these genes are here referred to as thin filament myopathies. For a schematic of the thin filament and its associated proteins, see Figure 1. Patients with thin filament myopathy suffer from muscle weakness, but the underlying mechanisms are poorly understood.

The thin filament is a major constituent of the sarcomere, the smallest contractile unit in muscle, and is essential for force generation; its length determines the overlap between the thin and thick filament, and thereby the number of force-generating interactions that can be formed between actin and myosin. In healthy human muscle, the length of the thick filament is 1.6 μm and that of the thin filament is regulated at 1.1 to 1.3 μm .⁷ Accordingly, force depends on sarcomere length, with increasing force as the overlap between thick and thin filaments increases (up to a sarcomere length of $\sim 2.6 \mu\text{m}$; ie, the ascending limb of the force–sarcomere length relation), and decreasing force at longer sarcomere lengths as the overlap between thick and thin filaments decreases (ie, the descending limb; Fig 2A^{8,9}). Hence, appropriate length of the thin filament is important for muscle fiber strength; a shorter length causes lower force generation by shifting the descending limb of the force–sarcomere length relation downward.

Whether mutations in genes implicated in thin filament myopathy contribute to force loss by affecting thin filament length is unclear. Mouse models with mutations in *Neb* exhibit a shorter thin filament length associated with lower force generation that becomes more prominent as sarcomere length increases.^{10–12} Preliminary studies on a small number of human biopsies were largely in agreement with this observation.^{13,14} Whether these findings translate to a large group of patients, and whether shorter thin filament length is a general mechanism underlying force loss in thin filament myopathies with a variety of different gene defects, is unknown.

Therefore, we studied muscle fibers from 51 patients with thin filament myopathy caused by mutations in *NEB*, *ACTA1*, *TPM2*, *TPM3*, *TNNT1*, *KBTBD13*, *KLHL40*, and *KLHL41*. In these fibers, we determined the sarcomere length-dependence of force, a functional assay that provides insight into the contractile strength of muscle fibers as well as the length of the thin filaments. In a novel, conditional *Neb* knockout mouse model that recapitulates thin filament myopathy—including shorter thin filament length—we studied whether muscle possesses mechanisms that compensate for shorter thin filament length, and we specifically focused on the addition of sarcomeres in series.

Subjects and Methods

Muscle Biopsies

Ethical approval for the use of muscle specimens remaining from diagnostic procedures or obtained during clinically indicated surgical procedures was obtained from the human research ethics committees of Boston Children's Hospital institutional review board (03-08-128R; *NEB*-1–*NEB*-3; *ACTA1*-1–*ACTA1*-4; *KLHL40*-1; *KLHL41*-1), Children's Hospital at Westmead (10/CHW/45; *ACTA1*-5–*ACTA1*-13; *TPM3*-1–*TPM3*-10; *TPM2*-1–*TPM2*-3; healthy controls [CTRL] 1–8), Neuromuscular and Neurogenetic Disorders of Childhood Section, National Institutes of Health, Bethesda, Maryland (*TPM3*-11–12), Pitié-Salpêtrière Hospital Group in Paris (DC-2012-1693; *NEB*-4–*NEB*-7, *NEB*-9, *ACTA1*-14), Radboud University Medical Center in Nijmegen (*KBTBD13*-1–*KBTBD13*-10), Children's Hospital of the University of Helsinki (6/E7/2005; *NEB*-8), and Hadassah Medical Center in Jerusalem (0421-14-HMO; *TNNT1*-1). Supplementary Table 1 shows the clinical and genetic data of the subjects who were biopsied. From all participants, written informed consent was obtained for genetic testing and biobanking of muscle.

CTRL 1–8 are control subjects with no medical history. All biopsies were stored frozen and unfixed at -80°C until use.

Permeabilized Muscle Fiber Mechanics

Small strips dissected from the muscle biopsies were permeabilized overnight as described previously.¹³ This procedure renders the membranous structures in the muscle fibers permeable, which enables activation of the myofilaments with exogenous calcium. Preparations were washed thoroughly with relaxing solution and stored in 50% glycerol/relaxing solution at -20°C . Small muscle bundles (cross-sectional area = $\sim 0.07\text{mm}^2$, in case of atrophic fibers in patients) and single muscle fibers (control subjects and mildly affected

patients) were dissected from the permeabilized strips, and were mounted using aluminum T-clips between a length motor (ASI 403A; Aurora Scientific, Aurora, Ontario, Canada) and a force transducer element (ASI 315C-I, Aurora Scientific) in a single fiber apparatus (ASI 802D, Aurora Scientific) that was mounted on the stage of an inverted microscope (Axio Observer A1; Carl Zeiss, Oberkochen, Germany). Sarcomere length was set using a high speed VSL camera and ASI 900B software (Aurora Scientific). Mechanical experiments were performed at incremental sarcomere lengths: 2.0, 2.2, 2.5, 2.8, 3.2, and 3.5 μm . Fiber width and diameter were measured at 3 points along the fiber, and the cross-sectional area was determined assuming an elliptical cross section. Three different types of bathing solutions were used during the experimental protocols: a relaxing solution (100mM N,N-Bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid [BES], 6.97mM ethyleneglycoltetraacetic acid [EGTA], 6.48mM MgCl_2 , 5.89mM Na_2 -adenosine triphosphate [ATP], 40.76mM K-propionate, 14.5mM creatine phosphate), a preactivating solution with low EGTA concentration (100mM BES, 0.1mM EGTA, 6.42mM MgCl_2 , 5.87mM Na_2 -ATP, 41.14mM K-propionate, 14.5mM creatine phosphate, 6.9mM hexamethylenediaminetetraacetic acid), and an activating solution (100mM BES, 7.0mM Ca-EGTA, 6.28mM MgCl_2 , 5.97mM Na_2 -ATP, 40.64mM K-propionate, 14.5mM creatine phosphate). The temperature of the bathing solutions was kept constant at 20°C using a TEC controller (ASI 825A, Aurora Scientific). To prevent rundown of force during the protocol, muscle preparations were mounted in a relaxation solution at 18°C. Subsequently, the muscle preparations were preactivated by switching to an activation solution at 1°C. In that way, fibers were loaded with calcium, but no force was generated. By rapid switching to an activation solution at 20°C, the fibers were activated and force was generated. This procedure minimizes the duration of activation and force rundown. After the force plateau was reached, relaxation was induced by switching back to the bath with relaxation solution.^{15,16}

Myosin Heavy Chain Isoform Analysis

A specialized sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was used to determine the myosin heavy chain isoform composition of the muscle fiber preparations that we used in our contractility experiments.¹³ In brief, muscle fibers were denatured by boiling for 2 minutes in SDS sample buffer and electrophoresis was performed for 24 hours at 15°C and a constant voltage of 275Vt using a 4% stacking gel (pH = 6.7), and a 7% separating gel (pH = 8.7) with 30% glycerol (vol/ vol). Finally, the gels were silver-stained, scanned, and analyzed with One-Dscan EX (Scanalytics, Rockville, MD) software.

Nebulin Protein Levels

To assess nebulin protein levels, muscle samples from CTRL (n = 5) and NEB (n = 7) were homogenized and analyzed on 1% agarose electrophoresis gels, as previously described.¹⁷ To prevent protein degradation, all buffers contained protease inhibitors (phenylmethylsulfonyl fluoride, 0.5mM; leupeptin, 0.04mM; E64, 0.01mM). Gels were scanned and analyzed with One-Dscan EX software. The integrated optical density of nebulin and myosin heavy chain was determined. For Western blot analysis, 1- or 2-color infrared Western blots were scanned (Odyssey Infrared Imaging System; LI-COR Biosciences, Lincoln, NE) and the images were analyzed with One-Dscan EX software.

Confocal Microscopy

Small muscle strips were dissected and permeabilized as described above in the Permeabilized Muscle Fiber Mechanics section. Immunolabeling and confocal scanning laser microscopy were performed as described previously.¹² In brief, muscle bundles were stretched and fixed on a glass slide, and incubated with the following antibodies: Alexa Fluor 488 conjugated phalloidin (A12379; Invitrogen, Carlsbad, CA) to stain the thin filament; and a cocktail of primary antibodies against slow skeletal myosin heavy chain (ab11083; Abcam, Cambridge, MA) and fast skeletal myosin heavy chain (ab51263, Abcam) followed by Alexa Fluor 594 (goat antimouse, Invitrogen) as secondary antibody to stain the thick filament. Images were captured using a Leica (Wetzlar, Germany) DM IRE2 Confocal Laser Scanning Microscope. From the acquired images, line scans were obtained using ImageJ software (National Institutes of Health, Bethesda, MD). For phalloidin line scans, the half width at half maximum was used to indicate thin filament length. Thick filament length was measured from myosin line scans (full width at half maximum).

Sarcomeres in Series in a Conditional Nebulin Knockout Mouse Model

For the creation of conditional nebulin knockout mice, see Li and coworkers.¹⁸ In brief, for conditional nebulin knockout mice, a targeting vector was made with loxP sites inserted downstream of exon 3 (which contains the start codon for nebulin and corresponds to exon 1 of Bang and coworkers¹¹) and in the 5' untranslated region of exon 2 upstream of the ATG. Floxed mice were bred to a *MCK-Cre* strain (#6475; Jackson Laboratory, Bar Harbor, ME) that expresses Cre recombinase under control of the muscle creatine kinase (MCK) promoter that is expressed in striated muscle.¹⁹ Genotyping was used to determine the presence of the *MCK-Cre* transgene (following the protocol provided by Jackson Laboratory) and the floxed nebulin allele. Mice homozygous for the floxed nebulin allele were bred with mice that were hemizygous for *MCK-Cre* and heterozygous for the floxed allele (*MCKCre*⁺, *Neb*^{+/*flox*}). Off-spring that was hemizygous for *MCK-Cre* and homozygous for the floxed allele (*MCKCre*⁺, *Neb*^{flox/flox}) was deficient in nebulin and is referred to as *Neb*^{-/-}. We used as control offspring that had nebulin wild-type allele and that was either *MCK-Cre* positive or negative. All animal experiments were approved by the University of Arizona Institutional Animal Care and Use Committee and followed the US National Institutes of Health "Using Animals in Intramural Research" guidelines for animal use.

Similar to the methods described above for human muscle fibers, permeabilized mouse fibers were activated with exogenous calcium at incremental sarcomere lengths to investigate the force–sarcomere length relation, and confocal microscopy was used to determine thin and thick filament length.

In addition, small permeabilized strips were isolated from tendon to tendon of mouse soleus muscle and were mounted in a single fiber apparatus (ASI 802D, Aurora Scientific) as described above in the Permeabilized Muscle Fiber Mechanics section. The sarcomere length was set at 2.5 μm using a high-speed VSL camera and ASI 900B software (Aurora Scientific). Muscle fiber length was determined from tendon to tendon. Muscle length was divided by the mean sarcomere length to provide the number of sarcomeres in series. This

number was normalized to tibia length (note that tibia length was not different between *Neb*^{-/-} and *Neb*^{+/+} mice.)

To determine the sarcomere length at the muscle length that generates optimal force, intact left soleus muscles were quickly dissected from tendon to tendon and, using silk suture, mounted vertically in a tissue bath between a dual-mode lever arm (300C, Aurora Scientific) and a fixed hook. Soleus muscles were chosen because of their well-defined tendons and their small size, which facilitates oxygenation. The muscle was bathed in continuously oxygenated (95% O₂–5% CO₂) mammalian Ringer solution with pH = 7.40. The temperature of the solution was maintained at 30°C during the experiment. The muscle was stimulated by field stimulation with platinum plate electrodes placed in close apposition to the muscle. Optimal muscle length was determined by stimulating the muscle maximally at incremental muscle lengths, until maximal force was achieved (pulse duration = 200 microseconds). Then, muscle length was measured from tendon to tendon, the muscle was chemically fixed at optimal length, and sarcomere length was measured with optical microscopy.

Statistical Analyses

Data are presented as mean ± standard error of the mean. For statistical analyses, *t* tests were used. A probability value < 0.05 was considered statistically significant.

Results

Muscle fibers were isolated from biopsies of CTRL (n = 8) and patients (n = 51) with mutations in *NEB* (n = 9), *ACTA1* (n = 14), *TPM3* (n = 12), *TPM2* (n = 3), *TNNT1* (n = 1), *KBTBD13* (n = 10), *KLHL40* (n = 1), and *KLHL41* (n = 1; for patient characteristics, see Supplementary Table 1). From each biopsy, 3 to 9 muscle fiber preparations were permeabilized and maximally activated with exogenous calcium. Force was measured at incremental sarcomere lengths (2.0–3.5 μm) to investigate the sarcomere– length dependency of force.

Maximal Force Generation Is Lower in Muscle Fibers from Thin Filament Myopathy Patients

To compare the contractile strength of muscle fibers from patients—which typically are atrophic—to those from control subjects, the generated forces were normalized to the fibers' cross-sectional area (ie, tension). Hence, maximal active tensions of muscle fibers are independent of the diameter of the fibers and reflect the intrinsic contractile capacity of the myofilament proteins. Lower maximal active tension was observed in all gene cohorts, when compared to CTRL muscle fibers (Table). Note that the protein product of *TPM3* is expressed predominantly in slow-twitch fibers, and accordingly, slow-twitch fibers from patients with mutations in this gene had lower maximal active tension, whereas in fast-twitch fibers maximal active tension was similar to that of fibers from CTRL. Thus, muscle fibers from patients with thin filament myopathy exhibit lower force generation, with the most pronounced force loss in patients carrying *NEB*, *ACTA1*, and *KLHL40* mutations.

The Force–Sarcomere Length Relation Is Shifted Downward in Muscle Fibers from Patients with Mutations in *NEB* and *ACTA1*

Force at incremental sarcomere lengths was fitted using a 2nd order polynomial.²⁰ This yielded 3 parameters that describe the force–sarcomere length relation: the sarcomere length at which maximum force is generated (SL_{opt}), the sarcomere length at which 50% of maximum force is generated (SL_{50}), and the sarcomere length at which the fit crosses the x-axis, thus no force is generated (SL_x ; see Fig 2A). The fits in Figure 2B–I represent the mean of the fits of the individual patients; data from individual subjects are shown in Supplementary Table 2. In *NEB* patients, a downward shift of the descending limb of the force–sarcomere length relation was observed, which was accompanied by a significantly shorter SL_{opt} ($2.41 \pm 0.04 \mu\text{m}$ in patients vs $2.61 \pm 0.02 \mu\text{m}$ in controls), SL_{50} ($3.39 \pm 0.05 \mu\text{m}$ in patients vs $3.58 \pm 0.03 \mu\text{m}$ in controls), and SL_x ($3.85 \pm 0.07 \mu\text{m}$ in patients vs $4.02 \pm 0.03 \mu\text{m}$ in controls). Accordingly, force loss was most prominent at long sarcomere lengths, with the force deficit doubling across the operating sarcomere length range in human skeletal muscle ($\sim 2.9 \mu\text{m}$ [87% of CTRL] to $3.6 \mu\text{m}$ [43% of CTRL]²¹; dashed area and right y-axis in Fig 2B). These findings suggest that patients with *NEB* mutations have shorter thin filament lengths.

A novel finding of this study is that mutations in *ACTA1*, encoding skeletal muscle alpha-actin, the main component of the thin filament, can also cause a significant downward shift of the descending limb of the force–sarcomere length relation, with significantly shorter SL_{50} ($3.47 \pm 0.02 \mu\text{m}$ in patients vs $3.58 \pm 0.03 \mu\text{m}$ in controls) and SL_x ($3.89 \pm 0.03 \mu\text{m}$ vs $4.02 \pm 0.03 \mu\text{m}$, patients vs controls; see Fig 2C). Note that fibers from some *NEB* patients (*NEB-1*, *NEB-4*, *NEB-5*, *NEB-8*) and some *ACTA1* patients (*ACTA1-5*, *ACTA1-6*, *ACTA1-9*, *ACTA1-10*) had force–sarcomere length relations that overlapped with controls (see Supplementary Table 2 and Fig 3A).

For the other thin filament genes that were investigated in this study—*TPM3*, *TPM2*, *TNNT1*, *KBTBD13*, *KLHL40*, and *KLHL41*—no changes in the force–sarcomere length relation were observed (see Fig 2D–H and). For *KLHL40*, *KLHL41*, and *TNNT1* only 1 biopsy representing each genotype was available for contractility experiments; thus, these findings should be interpreted with caution.

Confocal Microscopy Reveals Shorter Thin Filaments in Patients with Mutations in *NEB* and *ACTA1*

Next, we aimed to confirm that the downward shift of the descending limb of the force–sarcomere length relation in patients with mutations in *NEB* and *ACTA* was caused by shorter thin filament length (note that a downward shift of this relation can be caused by reduced length of the thin and/or thick filament). Therefore, we measured thin and thick filament length by confocal microscopy in muscle fibers from *NEB* and *ACTA1* patients exhibiting SL_{50} values that were in the range of control values (*NEB-1* and *ACTA1-6*) and in patients who had values that were markedly below this range (*NEB-2* and *ACTA1-1*; see Fig 3A–C). Muscle fibers of *NEB-2* and *ACTA1-1* had shorter thin filament lengths than CTRL ($0.94 \pm 0.12 \mu\text{m}$ in *NEB-2* and $0.96 \pm 0.06 \mu\text{m}$ in *ACTA1-1* vs $1.35 \pm 0.01 \mu\text{m}$ in CTRL), whereas thick filament lengths were comparable to CTRL ($1.62 \pm 0.02 \mu\text{m}$ in *NEB-2*

and $1.56 \pm 0.03 \mu\text{m}$ in *ACTA1-1* vs $1.61 \pm 0.03 \mu\text{m}$ in CTRL). As expected, in muscle fibers of *NEB-1* and *ACTA1-6* normal thin filament lengths ($1.32 \pm 0.02 \mu\text{m}$ in *NEB-1* and $1.28 \pm 0.05 \mu\text{m}$ in *ACTA1-6*) and thick filament lengths ($1.55 \pm 0.06 \mu\text{m}$ in *NEB-1* and $1.55 \pm 0.02 \mu\text{m}$ in *ACTA1-6*) were observed. Thus, confocal microscopy reveals that thick filament length was not altered in any of the samples. This indicates that the downward shift of the descending limb of the force sarcomere length relation was caused by shorter thin filament length.

A Mouse Model for Nebulin-Based Thin Filament Myopathy Suggests a Compensatory Mechanism for Shorter Thin Filaments in Muscle

We addressed whether muscle adds more sarcomeres in series to compensate for the sarcomere length-dependent loss of force. Adding more sarcomeres in series would blunt the effect of shorter thin filament lengths, as at a given muscle fiber length the fibers operate at a shorter sarcomere length, a length closer to their optimal length. Investigating the number of sarcomeres in series in muscle fibers of patients poses considerable technical and ethical difficulties. Therefore, we used the recently developed conditional nebulin knockout (*cNeb^{-/-}*) mouse, a model that represents the most frequently affected gene in thin filament myopathy, and that phenocopies several main features of human disease.¹⁸ (Note that muscle fibers from thin filament myopathy patients with *NEB* mutations were also nebulin-deficient; Supplementary Table 3.) This model enabled for the first time study of muscle adaptations in mature, nebulin-deficient muscle (previous *Neb*-mouse models had a lifespan of maximally 3 weeks¹⁰⁻¹²). As expected, fibers isolated from soleus muscle of adult *cNeb^{-/-}* mice revealed a downward shift of the descending limb of the force–sarcomere length relation and confocal microscopy confirmed that the observed downward shift was caused by shorter thin filaments (Fig 4A–C). Importantly, soleus muscle fibers of *cNeb^{+/+}* mice contained more sarcomeres in series ($7,010 \pm 176$ sarcomeres from tendon to tendon) compared to muscle of *cNeb^{+/+}* mice ($5,903 \pm 209$ sarcomeres from tendon to tendon; see Fig 4D). Note that tibia length was comparable between *cNeb^{+/+}* and *cNeb^{-/-}* mice.

The addition of sarcomeres in series should enable muscle of *cNeb^{-/-}* mice to operate at a shorter sarcomere length, a length closer to their optimal sarcomere length. To investigate whether muscle of *cNeb^{-/-}* mice operates at a shorter sarcomere length, whole intact soleus muscle was electrically activated and the optimal muscle length for force generation was determined. These studies revealed that the muscle length at optimal force generation was not different between *cNeb^{+/+}* and *cNeb^{-/-}* mice, and that at this muscle length the sarcomere length was shorter in *cNeb^{-/-}* than in *cNeb^{+/+}* ($2.51 \pm 0.04 \mu\text{m}$ vs $2.80 \pm 0.03 \mu\text{m}$, respectively; see Fig 4E). Thus, in *cNeb^{-/-}* mice, force loss caused by shorter thin filament lengths is compensated for by increasing the number of sarcomeres in series so that the muscle can operate at shorter sarcomere lengths and the amount of overlap between the thin and thick filaments is partly restored (summarized in Fig 4F).

Discussion

Ten genes have been implicated in thin filament myopathy. Studies focusing on the effects of specific mutations in these genes on sarcomere function are largely lacking, mainly because

patient biopsies have been limited in size and number. However, improved insight into genotype–sarcomeric phenotype correlations is essential for the development of targeted treatment strategies. Therefore, here we investigated the contractile strength of muscle fibers in 51 patients, covering 8 of the 10 implicated genes. Lower force generation was observed in muscle fibers from patients of all genotypes. Shorter than normal thin filament lengths were associated with lower force generation in patients with thin filament myopathy, but only in those who harbor specific mutations in *NEB* and *ACTA1*. A conditional *Neb* knockout mouse model, which recapitulates thin filament myopathy, revealed a compensatory mechanism in muscle; force loss due to shorter thin filaments was counteracted by increasing the number of sarcomeres in series.

Mutation-Specific Effects on Thin Filament Length

ACTA1 and *NEB* are the most frequently mutated genes in nemaline myopathy, the most common type of thin filament myopathy.²² Consequently, shortened thin filaments are likely to be a phenotype that contributes to lower force generation in a large number of patients with thin filament myopathy. The effect of shorter thin filaments on force development is sarcomere- and muscle length-dependent, with a more pronounced effect at greater lengths. This is illustrated in Figure 2B, where the *in vivo* sarcomere length range (~2.9–3.6 μm ²¹) is depicted; in patients with *NEB* mutations, the force deficit at a sarcomere length of 3.6 μm doubles compared to the deficit at a sarcomere length of 2.9 μm . This implies that muscle weakness in patients with mutations in *NEB* or *ACTA1* is more pronounced at larger joint angles, where muscle length is long. Whether this occurs in thin filament myopathy patients remains to be studied.

As some patients with *NEB* and *ACTA1* mutations, as well as the patients with mutations in *TPM3*, *TPM2*, *TNNT1*, *KBTBD13*, *KLHL40*, and *KLHL41*, displayed significant force deficits without a downward shift of the force–sarcomere length relation, other mechanisms must be at play in these patients. Previous work on animal models and on biopsies from patients with *NEB*, *ACTA1*, *TPM3*, and *TPM2* showed that qualitative changes in actin–myosin interactions as well as myofibrillar disarray are important contributors to lower force generation.^{6,12,23–28} Similarly, not all patients with mutations in *NEB* and *ACTA1* had shorter thin filament lengths; muscle fibers of some patients (*NEB*-1, *NEB*-4, *NEB*-5, *NEB*-8, *ACTA1*-5, *ACTA1*-6, *ACTA1*-9, *ACTA1*-10) had force–sarcomere length relations that overlapped with controls (see Supplementary Table 2 and Fig 3A). Thus, within the *NEB* and *ACTA1* cohorts, effects on thin filament length are mutation-specific. Although speculative, one could argue that mutations in *ACTA1* that reduce the binding strength between actin monomers are more likely to impact thin filament length than mutations that occur elsewhere. Similarly, although nebulin is important for thin filament length regulation,^{10,11} mutations in *NEB* that do not significantly affect its protein level or binding affinity of capping proteins might not disturb the regulation of thin filament length.¹⁴ Investigating mutation-specific effects on thin filament length will be an interesting topic for future studies.

In summary, because *ACTA1* and *NEB* are the most frequently mutated genes in thin filament myopathies,²² shortened thin filament length is likely to be a phenotype that

contributes to lower force generation in a large number of patients with thin filament myopathy.

Addition of Sarcomeres in Series to Compensate for Shorter Thin Filament Length

To date, it has been unknown whether and how muscle responds to shorter thin filament lengths. To identify such response, we used the recently developed conditional nebulin knockout (*cNeb*^{-/-}) mouse, a model that closely phenocopies human thin filament myopathy¹⁸ and, importantly, that made study of muscle adaptations in mature nebulin-deficient muscle possible for the first time. Previous *Neb*-mouse models had a maximal lifespan of 3 weeks, and consequently growth retardation and delays in muscle development could preclude meaningful explorations of response mechanisms. Adult *cNeb*^{-/-} mice had muscle fibers with shorter thin filament lengths, and consequently, in isolated muscle fibers the optimal sarcomere length for force production was shorter (see Fig 4A–C). However, we observed that *cNeb*^{-/-} mice increased the number of sarcomeres in series, and that as a result the optimal muscle length for force generation in intact muscle was comparable to *cNeb*^{+/+} mice; the addition of sarcomeres in series allowed the muscles to operate at a shorter sarcomere length (see Fig 4D–F), a length closer to their optimal sarcomere length. Adapting the number of sarcomeres in series to compensate for the changes in thin filament length suggests the existence of a compensatory mechanism in muscle. The molecular mechanisms that underlie this remain to be revealed, but might include proteins that sense suboptimal filament overlap, and in response produce more sarcomeric proteins. Previous work on animal models suggested that IGF-1, Akt, and cytosolic calcium mediate the addition of sarcomeres in series.^{29–31} Whether these mediators are players in sensing optimal filament overlap and are involved in the regulation of sarcomeric protein expression remains to be elucidated. Future studies should address whether this compensatory mechanism is also present in human diseased muscle and whether active stretching of muscle stimulates the addition of sarcomeres in series, as observed in rodents.³²

Study Limitations

The mutations in some of the genes that are implicated in thin filament myopathy are rare, which precludes the inclusion of large numbers of patients. We were able to study only 1 patient with a mutation in *KLHL40*, 1 with a mutation in *KLHL41*, and 1 with a mutation in *TNNT1*, and the force–sarcomere length relations in muscle fibers of these 3 patients were comparable to that in fibers of control subjects. Caution is warranted when drawing conclusions from this small sample size, and future studies on more patients with mutations in these genes should test whether thin filament length is unaffected. Second, muscle fibers in biopsies of several patients were very small, which precluded studies on individual fibers and forced us to use multifiber preparations. Previous work from our group revealed that the maximal active tension determined in single fibers is comparable to that determined in multifiber preparations.³³ Thus, it is unlikely that our results are affected by using both single and multifiber preparations.

Summary

We investigated a large cohort of patients covering the majority of genes that are implicated in thin filament myopathy. Lower force generation at the sarcomere level was observed in

muscle fibers from patients of all genotypes that were studied. Shorter than normal thin filament length contributes to the impaired force generation in patients with thin filament myopathy, but only in those who harbor specific mutations in *NEB* or *ACTA1*. Findings in a conditional *Neb* knockout mouse model suggest that in thin filament myopathy patients adding more sarcomeres in series might be a novel direction in counteracting force loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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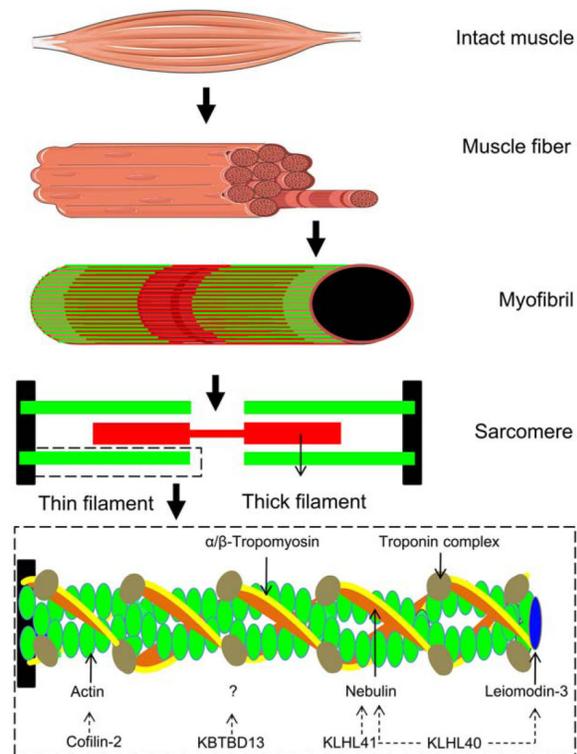
We thank the patients and their families for participating in this research and contributing to progress toward understanding the thin filament myopathies; L. Swanson for assistance ascertaining and enrolling patients with thin filament myopathies in Boston; and S. Conijn and C. Saripalli for their help with the revision experiments.

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**FIGURE 1.**

Schematic of the skeletal muscle thin filament. The thin filament is an essential structure in the sarcomere, the smallest contractile unit in skeletal muscle. The actin-based backbone of the thin filament is decorated with proteins that are involved in the regulation of thin filament length and in muscle activation. Tropomyosin and the troponin complex decorate the thin filament along its entire length and are important players in thin filament activation.³⁴ Actin, nebulin, and leiomodlin-3 are involved in both specifying the length of the thin filament and thin filament activation.^{6,34,35} Both cofilin-2 and the kelch proteins—KBTBD13, KLHL40, and KLHL41—are associated with thin filament proteins: cofilin-2 by regulating actin dynamics,³⁶ KLHL40 and KLHL41 by interacting with nebulin, and KLHL40 by stabilizing leiomodlin-3. The role of KBTBD13 has not yet been elucidated, but as it is required for the formation of a functional cullin-3 ubiquitin ligase complex, and as mutations in *KBTBD13* result in thin filament myopathy, it is hypothesized to be involved in stabilizing thin filament proteins.³⁷

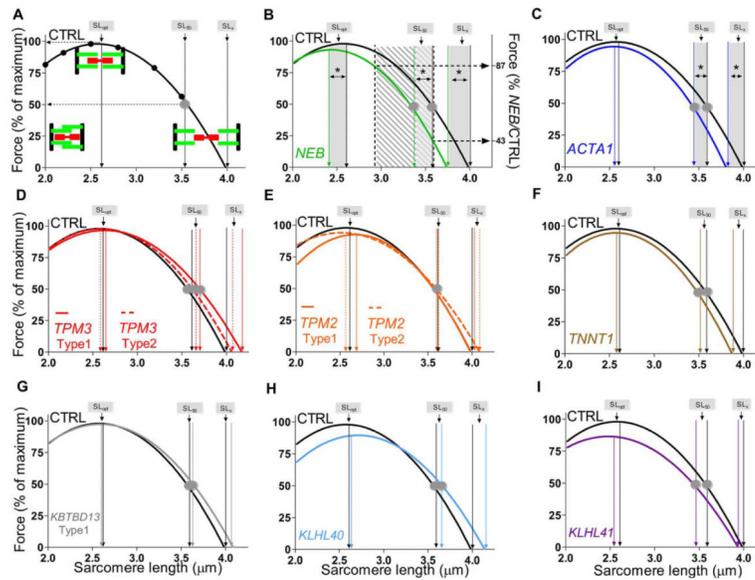


FIGURE 2.

The force–sarcomere length relation in muscle fibers from thin filament myopathy patients and control (CTRL) subjects. (A) The shape of the force–sarcomere length relation is determined by the amount of overlap between the thick and thin filament. Force at incremental sarcomere lengths is fitted using a 2nd order polynomial. This yields 3 parameters describing the force–sarcomere length relation: the sarcomere length at which maximum force is generated (SL_{opt}), the sarcomere length at which 50% of maximum force is generated (SL_{50}), and the sarcomere length at which the fit crosses the x-axis (SL_x). Data shown are from control subjects. The fits represent the mean of individual subjects per genotype. (B, C) *NEB* ($n = 9$) and *ACTA1* ($n = 14$) muscle fibers exhibit a downward shift of the descending limb of the force–sarcomere length relation compared to controls. Note that in patients with mutations in *NEB* (B), this results in a doubling of the force deficit (right y-axis) across the in vivo sarcomere length range (shaded area). (D–I) Muscle fibers from patients with mutations in *TPM3* ($n = 12$), *TPM2* ($n = 3$), *TNNT1* ($n = 1$), *KBTBD13* ($n = 10$), *KLHL40* ($n = 1$), and *KLHL41* ($n = 1$) exhibit a preserved force–sarcomere length relation. Note that the protein products of *TPM3* and *TPM2* are predominantly expressed in type I fibers and that these fibers exhibited normal force–sarcomere length relations. *t* tests were performed between genotype and CTRL; $p < 0.05$ was considered statistically significant (indicated by *asterisk*). For each biopsy, 3 to 9 muscle fibers were measured.

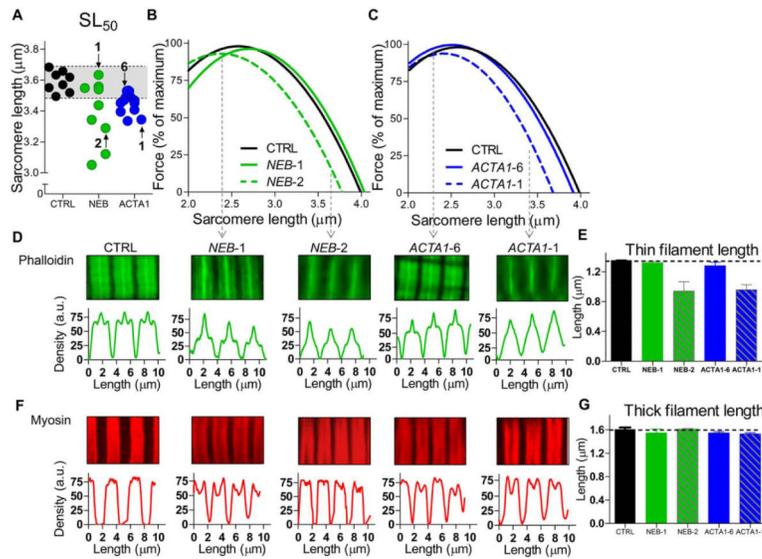
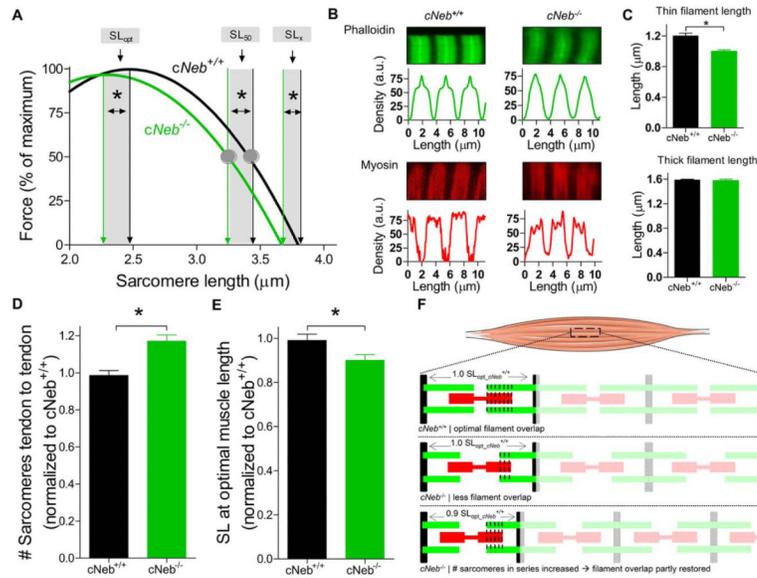


FIGURE 3. Confocal microscopy confirms shorter thin filament lengths in *NEB* and *ACTA1* patients. (A–C) Within the *NEB* and *ACTA1* cohorts, fibers of some patients exhibited force–sarcomere length relation characteristics that were within the range of control (CTRL) values (ie, *NEB-1*, *ACTA1-6*), whereas other patients (ie, *NEB-2*, and *ACTA1-1*) had values that were markedly below this range (normal range for sarcomere length at which 50% of maximum force is generated [SL₅₀] is in gray). (D–G) Fluorescein isothiocyanate–labeled phalloidin (green) was used to measure thin filament length, and antibodies against both slow and fast myosin heavy chain isoforms (red) were used to measure thick filament length. Thick filament length is normal in muscle fibers from CTRL, *NEB*, and *ACTA1* biopsies, whereas thin filaments are shorter in fibers that revealed a downward shift of the descending limb of the force–sarcomere length relation (*NEB-2* and *ACTA1-1*; n = 4 for CTRL, >5 images per genotype). *t* tests were performed between genotype and CTRL; *p* < 0.05 was considered statistically significant.

**FIGURE 4.**

A mouse model for nemaline myopathy compensates for shorter thin filaments by adding more sarcomeres in series. (A) Soleus muscle fibers from a mouse model that phenocopies human nemaline myopathy (conditional nebulin knockout, $cNeb^{-/-}$) revealed a downward shift of the descending limb of the force–sarcomere length relation compared to muscle fibers from $cNeb^{+/+}$ mice ($n = 4$ mice/group). (B, C) Confocal microscopy revealed shorter thin filaments in $cNeb^{-/-}$ mice. (D) $cNeb^{-/-}$ mice compensated for the force deficit caused by shorter thin filaments by increasing the number of sarcomeres in series ($n = 6$ mice/group). (E) At optimal length for force generation, intact soleus muscle of $cNeb^{-/-}$ mice operated at a shorter sarcomere length (SL) than $cNeb^{+/+}$ mice ($n = 20$ mice/group). (F) The addition of sarcomeres in series increases the amount of overlap between the thin and thick filaments and enables the fibers with shorter thin filaments to operate at a length closer to their optimal length. t tests were performed between $cNeb^{+/+}$ and $cNeb^{-/-}$ mice, $p < 0.05$ was considered statistically significant (indicated by asterisk). SL_{opt} = SL at which maximum force is generated; SL_{50} = SL at which 50% of maximum force is generated; SL_x = SL at which the fit crosses the x-axis.

TABLE

Functional Data of Thin Filament Myopathy Patients and Controls

Gene	Biopsies, No.	Maximal Active Tension, mN/mm ²	SL _{opt} , μm	SL ₅₀ , μm	SL _x , μm
CTRL	8	143 ± 16	2.61 ± 0.03	3.59 ± 0.02	4.03 ± 0.04
<i>NEB</i>	9	41 ± 11 ^a	2.41 ± 0.08 ^a	3.39 ± 0.07 ^a	3.85 ± 0.08 ^a
<i>ACTA1</i>	14	51 ± 7 ^a	2.57 ± 0.02	3.47 ± 0.02 ^a	3.89 ± 0.03 ^a
<i>TPM3</i> slow-twitch	11	86 ± 12 ^a	2.64 ± 0.04	3.67 ± 0.04	4.14 ± 0.05
<i>TPM3</i> fast-twitch	7	161 ± 5	2.62 ± 0.06	3.64 ± 0.06	4.10 ± 0.06
<i>TPM2</i> slow-twitch	2	56 ± 13 ^a	2.68 ± 0.22	3.61 ± 0.18	4.04 ± 0.17
<i>TPM2</i> fast-twitch	3	150 ± 15	2.56 ± 0.09	3.75 ± 0.07	4.27 ± 0.09
<i>TNNT1</i>	1	50 ± 3	2.60 ± 0.03	3.53 ± 0.12	3.98 ± 0.20
<i>KBTBD13</i>	10	73 ± 6 ^a	2.60 ± 0.04	3.65 ± 0.04	4.13 ± 0.06
<i>KLHL40</i>	1	5.0 ± 2	2.63 ± 0.13	3.66 ± 0.04	4.17 ± 0.07
<i>KLHL41</i>	1	68 ± 4	2.68 ± 0.07	3.60 ± 0.04	4.04 ± 0.05

Note that for *TNNT1*, *KLHL40*, and *KLHL41*, 1 biopsy was evaluated and therefore no statistical testing was performed.

See Supplementary Table 2 for mean values per individual subject.

CTRL = control; SL₅₀ = sarcomere length at which 50% of maximum force is generated; SL_{opt} = sarcomere length at which maximum force is generated; SL_x = sarcomere length at which the fit crosses the x-axis.

^aSignificantly different from CTRL ($p < 0.05$).