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KBTBD13 IS AN ACTIN-BINDING PROTEIN THAT MODULATES MUSCLE KINETICS

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ABSTRACT  (150 words)

The mechanisms that modulate the kinetics of muscle relaxation are critically important for muscle function. A prime example of the impact of impaired relaxation kinetics is nemaline myopathy caused by mutations in KBTBD13 (NEM6). In addition to weakness, NEM6 patients have slow muscle relaxation, compromising contractility and daily-life activities. The role of KBTBD13 in muscle is unknown, and the pathomechanism underlying NEM6 is undetermined. A combination of transcranial magnetic stimulation-induced muscle relaxation, muscle fiber- and sarcomere-contractility assays, low angle X-ray diffraction and super-resolution microscopy revealed that the impaired muscle relaxation kinetics in NEM6 patients are caused by structural changes in the thin filament, a sarcomeric microstructure. Using homology modeling, binding- and contractility assays with recombinant KBTBD13, novel Kbtbd13-knockout and Kbtbd13R408C-knockin mouse models and a GFP-labeled Kbtbd13-transgenic zebrafish model we discovered that KBTBD13 binds to actin – a major constituent of the thin filament - and that mutations in KBTBD13 cause structural changes impairing muscle relaxation kinetics. We propose that this actin-based impaired relaxation is central to NEM6 pathology.
INTRODUCTION
Regulation of muscle contraction is an intricate interplay between the actin-based thin filament and the myosin-based thick filament, both key components of the sarcomere, the smallest contractile unit in muscle (Fig.1A). During muscle activation, the number of force generating myosin molecules (i.e., cross bridges) rapidly builds and high forces are generated; during relaxation this number rapidly falls to bring force down and allow lengthening of passive muscle to occur. The kinetics of muscle activation have been widely studied but relaxation is much less well understood, despite its crucial importance for muscle contractility and disease (1–3). A prime example of the impact of impaired relaxation kinetics on muscle contractility is nemaline myopathy (NEM) caused by mutations in KBTBD13 (NEM6). In addition to weakness, NEM6 patients have slow muscle relaxation (4–6), which impairs their ability to perform fast movements, e.g. running or climbing the stairs (see Supplemental video 1-3), and to prevent themselves from falling when tripping.

NEM's are among the most common non-dystrophic congenital myopathies (7). So far, thirteen genes have been implicated: alpha-actin 1 (ACTA1)(8), alpha- and beta-tropomyosin (TPM3 and TPM2)(9, 10), nebulin (NEB)(11), leiomodin-3 (LMOD3)(21), troponin T (TNNT1)(12) and TNNT3(13), cofilin 2 (CFL2)(14), unconventional myosin 18B (MYO18B)(15), myopalladin (MYPN)(16), kelch family members 40 (KLHL40) and -41 (KLHL41)(17, 18), and kelch repeat and BTB (POZ) Domain Containing 13 (KBTBD13)(19).

Eleven out of these thirteen genes encode proteins that are associated with the actin-based thin filament, and recent studies revealed that thin filament dysfunction is a major contributor to muscle weakness in NEM(20–23). Hence NEM is considered a disease of the thin filament.

Interestingly, KBTBD13 has no established association with the thin filament (Fig.1B). In fact,
the localization and function of KBTBD13 are obscure(24), and, therefore, the patho-
mechanism underlying weakness and impaired relaxation kinetics of muscle in NEM6
patients is unknown. The goal of this study was to discover this pathomechanism. As the
vast majority of genes implicated in NEM encode thin filament-associated proteins, we
hypothesized that the impaired muscle relaxation kinetics in NEM6 patients are caused by
changes in thin filament structure and function.

To test this hypothesis, we applied techniques to study muscle relaxation at various levels of
organization, ranging from the patient in vivo to individual sarcomeres isolated from patient
biopsies. A combination of transcranial magnetic stimulation-induced in vivo muscle
relaxation studies, in vitro muscle fiber- and sarcomere-contracility assays, and low angle X-
ray diffraction and super-resolution microscopy revealed that the impaired muscle relaxation
kinetics in NEM6 patients is thin filament-based. Using homology modeling, binding- and
contractility assays with recombinant KBTBD13, novel Kbtbd13-knockout and Kbtbd13R408C-
knockin mouse models and a GFP-labeled Kbtbd13-transgenic zebrafish model we
discovered that KBTBD13 is an actin-binding protein and that mutations in KBTBD13 affect
the kinetics of muscle relaxation through effects on thin filament structure. Hence, this study
identifies (1) KBTBD13 as a regulator of skeletal muscle relaxation and (2) the patho-
mechanism underlying muscle dysfunction in NEM6 myopathy as thin-filament based
impaired muscle relaxation kinetics.
RESULTS

Lower muscle force and slower relaxation kinetics in NEM6 patients in vivo

Patient characteristics are shown in Table 1. In vivo muscle force and relaxation kinetics of deep finger flexors were determined using non-invasive transcranial magnetic stimulation (TMS) (25) (Fig. 2A). Maximal force was lower in NEM6 patients than in control subjects, both in males and in females (Fig. 2B). Because relaxation kinetics were similar between males and females, these data were pooled. Figure 2A (middle panel) shows a typical relaxation trace of a patient and control subject with marked slower muscle relaxation. The normalized peak relaxation rate was lower in NEM6 patients than in controls (Fig. 2C), and accordingly, the 75% relaxation time (time for force to drop by 25%) was longer in NEM6 patients than in controls (Fig. 2D). This demonstrates that the origin of slower muscle relaxation in NEM6 patients is myogenic.

Lower contractile force and slower relaxation kinetics in NEM6 muscle fibers

First, we studied whether changes in Ca$^{2+}$ handling proteins contributed to impaired muscle relaxation in NEM6. The level of SERCA1, a key protein involved in sarcoplasmic reticulum Ca$^{2+}$ handling in fast-twitch fibers, was not significantly different between NEM6 and control muscle (Supplemental Fig. 1A-B). SERCA1 protein levels and SERCA activity strongly correlated with the area of fast-twitch fibers in muscle biopsies of NEM6 patients (Supplemental Fig. 1C-E). The ratio of phospholamban (active form) over phosphorylated phospholamban (inactive form) was increased in NEM6 muscle biopsies (Supplemental Fig. 1F). As phospholamban slows SERCA activity, this might contribute to slower muscle relaxation in NEM6. Electron microscopy analyses showed no aberrations in the structure of the triads in muscle fibers of NEM6 patients.

Next, to study whether sarcomeric changes contribute to slower muscle relaxation kinetics we isolated permeabilized single muscle fibers from biopsies of NEM6 patients and activated these with exogenous Ca$^{2+}$. Patient characteristics are shown in Table 1. Fig. 3A-B shows...
electron microscopic images of muscle fibers from a control and a NEM6 patient, and Fig.3C-D show images of representative slow-twitch and fast-twitch NEM6 patient fibers that were used for mechanics. In NEM6 patients, the ratio of slow-twitch/fast-twitch fiber cross-sectional area was increased (Supplemental Fig.2A), indicating that the fibers used for mechanics reflect the fiber size distribution in the whole biopsy as determined by histology (Supplemental Fig. 2B-C). Next, fibers were exposed to incremental Ca\textsuperscript{2+} concentrations and the resulting forces were recorded. Maximal absolute force (i.e. force at pCa 4.5) was lower in fast-twitch fibers of NEM6 patients compared to those of control subjects, but not in slow-twitch NEM6 fibers compared to those of controls (Supplemental Fig.2D-E, left panels). To correct for differences in fiber size, force was normalized to the cross-sectional area of the fiber (i.e. tension). In NEM6 patients, maximal tension of both slow-twitch and fast-twitch fibers was significantly lower than in fibers of control subjects (tension reduced by ~50%; Fig.3E-F). The lower maximal active tension was not caused by shorter thin filament lengths: the sarcomere length dependence of maximal tension was not different between NEM6 and control fibers (Supplemental Fig.2F). This finding was in line with the unaltered thin filament length in NEM6 fibers, as determined by super-resolution STED microscopy (Supplemental Fig.2F). The calcium sensitivity of both slow-twitch and fast-twitch fibers was increased in NEM6 patients, as reflected by the higher pCa\textsubscript{50} (Supplemental Fig.2D-E, right panels). Note that we observed no changes in the passive stiffness of NEM6 muscle fibers (Supplemental Fig.3), suggesting that titin does not contribute to the muscle stiffness experienced by NEM6 patients.

Importantly, the kinetics of relaxation after maximal activation were markedly slower in both slow-twitch and fast-twitch fibers of NEM6 patients when compared to control subjects. Figure 3G shows a typical relaxation trace of a patient’s and a control subject’s muscle fiber. The relaxation kinetics, reflected by the relaxation constant $K_{rel}$, were ~three-fold slower in NEM6 fibers (Fig.3H). Thus, our findings on permeabilized NEM6 muscle fibers suggest that
the mechanism contributing to slower muscle relaxation kinetics in NEM6 patients is at least partly sarcomere-based and independent of muscle fiber type.

**Lower contractile force and slower relaxation kinetics in NEM6 myofibrils**

Considering that a single fiber (diameter ~100 µm) is a collection of myofibrils (diameter ~1 µm; Fig.1A), contractile weakness of single fibers could be caused by ultrastructural damage or misalignment of myofibrils. Furthermore, the determination of relaxation kinetics in fibers could be confounded by the different size of the NEM6 fibers compared to control fibers (Supplemental Fig.2A-C), which impacts diffusion time of solutions. Therefore, we also measured the contractility of NEM6 myofibrils. Figure 3 A-B & I-J shows electron microscopic (EM) images of a control and a NEM6 muscle fiber (one myofibril is highlighted). The EM image of the NEM6 muscle fiber reveals that areas with myofibrillar damage as well as areas with preserved myofibrillar ultrastructure are present within one muscle fiber (note that nemaline rods were frequently observed in EM images of the NEM6 patients, for a high-magnification example, see Supplemental Fig.4). We isolated single myofibrils from the intact areas of NEM6 muscle fibers and measured their contractile function (experimental arrangement shown in Fig.3K). As shown in Fig.3L the maximal tension of myofibrils of NEM6 patients was lower than that of control subjects. Thus, in addition to myofibrillar damage, myofibrillar dysfunction contributes to fiber weakness in NEM6 patients. Importantly, whereas the activating kinetics were on average similar between NEM6 and control myofibrils (an example is shown in Fig.3M), the relaxation kinetics were slower in myofibrils of NEM6 patients than in those of control subjects (Fig.3N-O; note that Fig.3N shows the same myofibril as in Fig.3M). Thus, our findings on individual myofibrils indicate that slower sarcomeric relaxation kinetics contribute to slow muscle relaxation kinetics in NEM6 patients.

**Structural changes in the thin filament suggest a stiffer thin filament in NEM6 patients.**
As the findings from the fiber and myofibril studies point towards a sarcomere-based mechanism, we next determined whether structural changes in the thick and/or thin filament cause the slow relaxation kinetics. At the Advanced Photon Source (Argonne National Laboratories) low angle X-ray diffraction patterns were obtained from preparations in which twenty-eight fibers were mounted and aligned in one plane between two-halves of EM grids. This approach allowed for high quality equatorial and meridional diffraction patterns (for example, see Fig.4A). The spacing of the equatorial reflections indicated that lattice spacing was not different between NEM6 and control fibers ($d_{1,0}$; 40.76±0.37 vs. 40.95±0.45 nm, respectively) and the equatorial intensity ratios indicated that the position of myosin heads between the thick and thin filaments was not different between NEM6 and control fibers($I_{1,1}/I_{1,0}$; 0.71±0.13 vs. 0.68±0.05, respectively). The meridional reflections indicated no spacing changes in thick filament structure: both the myosin head displacement from the thick filament backbone ($M_3$-reflection: 14.53±0.02 nm for control and 14.46±0.03 nm for NEM6 fibers) and the thick filament backbone periodicity ($M_6$-reflection, Fig.4C) were comparable in fibers of NEM6 patients and those of controls. Reflections from the thin filament, however, were different between NEM6 and control fibers: the actin layer line 6 (ALL6) indicating the left-handed pitch of the thin filament helix was shorter in NEM6 than in control fibers (Fig.4D). Thus, low angle X-ray diffraction studies suggest that the actin-based thin filament is more tightly wound in NEM6 muscle fibers, a change that might reflect increased thin filament stiffness.

**Slower relaxation kinetics and stiffer thin filaments in muscle of Kbtbd13<sup>R408C</sup> mice.**

To further investigate the effect of the Dutch founder mutation KBTBD13<sup>R408C</sup> on muscle relaxation kinetics and thin filament stiffness, we engineered a Kbtbd13<sup>R408C</sup>-knockin (KI) mouse model (Fig.5A). Mice were studied at nine months after birth (this age was chosen as in patients the phenotype is slowly progressive).

**Basic characteristics of the model.** Homozygous mice were born at significantly higher than Mendelian ratio (Supplemental Fig.5A). Quantitative PCR showed that homozygous KI mice
expressed only mutant *Kbtbd13* and that heterozygous mice expressed ~50% of mutant and 50% of wt *Kbtbd13* (Supplemental Fig.5C). KI mice had higher body weight than wt mice (Fig.5B). Soleus and diaphragm muscle weights were higher in KI compared to wt mice (Supplemental Fig.5D). Picro-sirius red stained cryosections of m. soleus (representative images in Supplemental Fig.5E) showed a significantly increased area of extracellular matrix in KI compared to wt mice (14±1% vs. 12±1%, respectively). Soleus muscle of KI mice had a lower percentage of slow-twitch (type 1) fibers than wt mice (28±2% vs. 60±3%, respectively; Supplemental Fig.5E). The cross-sectional area of slow-twitch and fast-twitch (type 2A) fibers was larger in m. soleus of KI than of wt mice (Supplemental Fig.5F). Similar to NEM6 patients (Supplemental Fig.2C), in KI mice the cross-sectional area of slow-twitch fibers was larger than that of fast-twitch fibers (Supplemental Fig.5G). Gomori trichrome stained sections of m. soleus showed nemaline bodies in muscle fibers (Fig.5C), a key characteristic of nemaline myopathy. This finding was confirmed in fibers stained with phalloidin, showing actin-positive nemaline bodies (Fig.5D). Thin filament and thick filament length in muscle of KI mice was comparable to that in wt mice, as determined by super-resolution STED microscopy (Fig.5E-F). Thus, the pathology observed in *Kbtbd13*-KI mice resemble NEM6 pathology.

**Relaxation kinetics.** Maximal tetanic tension (150Hz) of m. soleus was comparable between KI and wt mice (Fig.5G), and the force-frequency curve was shifted to the left (Supplemental Fig.5H). Importantly, similar to observations in NEM6 patients, the relaxation kinetics of m. soleus after maximal tetanic stimulation were slower in KI mice than in wt mice (Fig.5H). After single twitch stimulation, relaxation kinetics were also slower in KI mice compared to wt mice (Supplemental Fig.5I). The level of SERCA1 was not significantly different in KI compared to wt muscle (Supplemental Fig.5J).

**Thin filament stiffness.** Next, we determined the structure of the thin and thick filaments in muscle of KI and wt mice. Low angle X-ray diffraction patterns were obtained from intact m. soleus in the relaxed state and at maximal tetanic activation. The meridional reflections indicated that in the relaxed state the structure of the thick filament was different in KI than in
wt muscle: the thick filament backbone periodicities (M6-reflection and the 2.8 nm-reflection; Supplemental Fig. K&L) were both shorter in KI muscle than in wt muscle. However, the relative extension of the thick filament during maximal tetanic activation was comparable between KI and wt muscle (Δ2.8 nm; Supplemental Fig.5M), suggesting a comparable thick filament stiffness.

Reflections from the thin filament were also different between KI and wt muscle. Similar to the observations in NEM6 muscle, the ALL6 reflection was shorter in KI than in wt muscle (Fig.5J). Importantly, working with intact mouse muscles allowed us to determine actin subunit spacings (i.e. 2.7 nm reflections, Fig.5K). This reflection could not be resolved in the X-ray diffraction patterns of permeabilized human muscle fibers (Fig.4A). We took advantage of this opportunity and observed that in relaxed muscle the actin sub-unit spacing was shorter in KI than in wt mice (Fig.5K), supporting our notion that the shortened ALL6 reflection indicates a more compact thin filament. Then, to study whether the thin filament is stiffer in KI mice, we analyzed the change in the 2.7 nm reflection during maximal tetanic activation. As shown in Fig.5L the change in this reflection (Δ2.7 nm) was reduced in KI muscle. Thus, this indicates that during activation the thin filament extends less in KI muscle than in wt muscle.

Next, we used the Δ2.7 nm data to calculate the longitudinal stiffness of the thin filaments. To this end, it is important to investigate how the isometric tension is distributed over the myofilaments in the sarcomere. Hence, an ultrastructural analysis was performed on electron micrographs of KI and wt m. soleus cross-sections. No significant difference was observed in myofibrillar fractional area in KI compared to wt muscle (inter-myofibrillar mitochondria: 3.6±0.1% vs. 2.4±0.4, respectively; subsarcolemmal mitochondria: 8.9±0.5% vs. 7.9±0.6%, respectively). We then calculated the force per individual thin filament in activated muscle. Briefly, the unit cell area of the myofilament lattice was calculated from the d1,0 equatorial spacing (in relaxed state: 34.2±0.1 vs. 32.7±0.2 nm, KI vs. wt, respectively) according to Millman(26), with the number of unit cells per muscle cross-section equaling the myofibril area (which was calculated using the mitochondrial area and the extracellular matrix area) divided by the unit cell area. Furthermore, the tension distribution along the thin and thick
filaments was determined from their lengths (which were comparable between KI and wt muscle; Fig.5E&F) and the overlap between them. For myofilament overlap we assumed a sarcomere length of 2.8 µm (based on Kiss and coworkers(27)). The force obtained per thin filament was significantly higher in KI muscle than in wt muscle (137.1±2.8 pN vs. 124.6±2.1 pN, respectively). Normalizing this force to the extension of the thin filament (Δ2.7 nm reflection) showed that the force required to stretch the thin filament was significantly increased in KI muscle compared to wt muscle (Fig.5M). Thus, thin filament stiffness is higher in muscle of KI mice.

**Normal relaxation kinetics in muscle of Kbtbd13-KO mice**

We also generated a Kbtbd13-knockout mouse model to study the effect of absence of Kbtbd13 on muscle relaxation kinetics. Similar to the mice of the Kbtbd13<sup>R408C</sup> KI model, mice were studied nine months after birth.

**Basic characteristics of the model.** Homozygous mice (KO; Supplemental Fig.6A) were born at close to Mendelian ratio (Supplemental Fig.6B). GeneArrayAnalyzer showed that Kbtbd13 mRNA levels were severely reduced in KO mice (Supplemental Fig.6C). KO mice had lower body weight than wt mice (Supplemental Fig.6D). Soleus muscle weights were lower in KO mice compared to wt mice (Supplemental Fig.6E), and quadriceps muscle weights were increased in KO mice.

**Relaxation kinetics.** The maximal tetanic tension (150Hz) of m. soleus was reduced in KO mice (Supplemental Fig.6F), and the force-frequency relation was comparable between KO and wt mice (Supplemental Fig.6G). The level of SERCA1 was not significantly different in KO compared to wt muscle (Supplemental Fig.5H). Importantly, the relaxation kinetics of both single twitches and maximal tetani were comparable between KO and wt mice (Supplemental Fig.6I, left and right panel, respectively). Thus, the relaxation kinetics of muscle of Kbtbd13-KO mice do not mimic those of Kbtbd13<sup>R408C</sup> KI mice and NEM6 patients.

**Relation between thin filament stiffness and sarcomere relaxation kinetics**
To evaluate whether changes in thin filament stiffness affect sarcomeric relaxation kinetics, simulations of paired compliant filaments were performed as described previously by Campbell(28) (for schematic, see Supplemental Fig.7A). Increasing the stiffness of the actin filaments slowed the rate of relaxation from 2.53 s⁻¹ to 4.37 s⁻¹ (Supplemental Fig.7B, lower right panel) without producing a substantial change in maximum isometric force (Supplemental Fig.7B, upper right panel). Thus, the combination of the results of the simulation experiments, the x-ray diffraction experiments and the contractility assays in patient biopsies and in mouse models suggest that mutations in \textit{KBTBD13} increase thin filaments stiffness, and that increased thin filament stiffness can slow sarcomeric relaxation kinetics.

\textbf{KBTBD13 binds to actin}

In order to examine the potential for KBTBD13 to bind to actin, we turned to homology modeling anticipating that structural features of the protein provide insight into potential interactions. Given the low sequence similarity between KBTBD13 and other proteins (< 30\%) three independent programs with different template starting structures were used for the modeling process. We note a high degree of structural similarity between the structured regions of our three resulting models (R.M.S.D. < 0.8 Å) that supports the relevance of our model. Similar to other proteins that contain repeating Kelch motifs, the resulting KBTBD13 homology model contains a beta propeller structure. The beta propeller is located in the C terminal portion, while the BTB motif adopts a more loosely defined zinc finger in the N-terminal region. Intriguingly, protein sequence analysis revealed similarities between KBTBD13 and the actin binding protein scruin, found in limulus sperm(29). To assess the similarities between the scruin and KBTBD13 beta propeller motifs, we also developed a homology model for the scruin protein. A different template structure was used for constructing the scruin homology model to avoid bias. We found several similarities between the beta propeller motif of KBTBD13 and scruin (Fig.6A, left & middle panel). In both beta propeller motifs the majority of cysteines are contained internally within the beta sheets. In
scruin, a single cysteine (Cys837) located in a loop at the surface of the beta propeller was found to be important for the interaction with filamentous actin(30). Importantly, KBTBD13 has a single cysteine (Cys367) located in a similar position relative to scruin Cys837 (Fig.6A, right panel). Scruin is known to bind to filamentous-actin, and Cys837 is important for this binding. These findings suggest that KBTBD13 might interact with actin. Strikingly, the location of three KBTBD13 mutations (I369M, K390N and R408C) are in close proximity to Cys367 (Fig.6A, left panel).

To test whether KBTDB13 binds to actin, we performed pull-down assays with full-length KBTBD13 and muscle lysates. The pulled down proteins were separated on SDS-PAGE gel (Fig.6B). One unique band was observed, at a height that corresponds to a molecular weight of 40-45kD. The proteins in this band were identified by mass spectrometry. Actin was one of the major proteins identified. Then, to validate whether KBTBD13 also binds to filamentous actin in vitro, and whether the most prevalent disease-causing mutation affects this binding, we expressed full-length KBTBD13 and KBTBD13R408C and performed a co-sedimentation assay with filamentous actin. The data demonstrate that both KBTBD13 and KBTBD13R408C bind to filamentous actin with comparable affinities (Fig.6C, left panel). As actin binding properties were not different between KBTBD13 and KBTBD13R408C we fitted a single curve using the data from both proteins (shared fit). The resulting binding curve showed a Kd of 3.25 ± 19.08 µM to actin, and a maximum binding of ~1.08 ± 1.92 µM KBTBD13 to 8 µM actin suggesting a stoichiometry of approximately one KBTBD13 molecule to eight actin molecules. Recombinant KBTBD13 also showed some sedimentation in the absence of actin (Fig.6C, right panel, P-), however, in the presence of actin (P+) greater amounts of KBTBD13 were consistently detected in the pellet and all data points are corrected for the amount of KBTBD13 sedimenting in the absence of actin at each concentration. GST alone did not sediment in the presence or absence of actin. Thus, our findings suggest that KBTBD13 is an actin binding protein.
We next determined the localization of KBTBD13 in muscle fibers. We were unable to detect KBTBD13 using antibodies (we tested commercially available antibodies and two custom-made antibodies). Therefore, we generated transgenic Kbtbd13-eGFP and Kbtbd13R408C-eGFP zebrafish. Note that in these fish the fibers display mosaic expression of KBTBD13, hence only few cells show labeling. Confocal microscopy analysis suggests that both KBTBD13 and KBTBD13R408C localize to the myofibrils, and the fluorescence pattern, throughout the sarcomere with a peak at the Z-disc, appears consistent with co-localization with actin (Supplemental Fig.8A-B). Then, to more accurately localize KBTDB13, we made use of APEX - a peroxidase enzyme that can be used to stain samples for electron microscopy. The peroxidase was coupled to a GFP-binding peptide that then allowed for electron microscopy localization of GFP-tagged Kbtbd13. We made a transgenic line of fish that contains the GFP-binding APEX protein and injected these with Kbtbd13-eGFP. As shown in Fig.6D, the electron microscopy-APEX data indicate that KBTBD13 localizes to the myofibrils. Within the myofibrils, the A-band (mainly myosin-based thick filaments) and the I-band (mainly actin-based thin filaments) show dark labeling, indicating that KBTBD13 binds to the thin filament and the thick filament. Furthermore, the z-discs, where thin filaments from adjacent sarcomeres overlap, show strong labeling. Note that to increase the contrast between KBTBD13-positive and -negative areas, the ‘normal’ EM image (Fig.6D, top left) was converted from gray scale to spectrum scale (Fig.6D, top right), with KBTBD13 in light blue. The electron microscopy images of transgenic Kbtbd13R408C-eGFP fish showed comparable localization as the transgenic Kbtbd13wt-eGFP fish (Supplemental Fig.8C). Thus, the data from these experiments support the confocal, pull-down, and co-sedimentation data that KBTBD13 binds to actin in the thin filaments of muscle.

**KBTBD13R408C increases the flexural rigidity of actin filaments**

To study whether binding of KBTBD13 directly affects the structure of thin filaments, we tested the effect of KBTBD13wt and KBTBD13R408C on the flexural rigidity (assessed by determining the persistence length) of actin filaments in solution. Fig.6E (left panel) shows a
typical image of actin filaments in solution (Supplemental video 4-6 show movies of actin filaments in solution). The results show that KBTBD13\textsubscript{wt} does not change the persistence length of actin filaments compared to that of actin filaments not exposed to KBTBD13\textsubscript{wt} (Fig.6E, right panel). However, KBTBD13\textsuperscript{R408C} significantly increases the persistence length of actin filaments (Fig.6E, right panel). These findings show that binding of KBTBD13\textsuperscript{R408C} directly increases the stiffness of actin filaments.

**KBTBD13\textsuperscript{R408C} slows relaxation of human muscle fibers**

To study whether slower relaxation kinetics in NEM6 muscle fibers are a direct effect of mutant KBTBD13, we incubated permeabilized quadriceps single muscle fibers from control subjects and NEM6 patients with either KBTBD13\textsubscript{wt} or KBTBD13\textsuperscript{R408C} and studied contractile function (Fig.6F, left panel). In fibers from NEM6 patients, the maximal forces were comparable between groups (108±20 mN/mm\textsuperscript{2} for fibers incubated with KBTBD13\textsubscript{wt} and 120±17 mN/mm\textsuperscript{2} for fibers incubated with KBTBD13\textsuperscript{R408C}). Incubation with KBTBD13\textsubscript{wt} slightly increased the relaxation kinetics compared to incubation with KBTBD13\textsuperscript{R408C} (Fig.6F, right panel), however, this difference did not reach significance. In fibers from control subjects, maximal forces were also comparable between groups (125±9 mN/mm\textsuperscript{2} for fibers incubated with KBTBD13\textsubscript{wt} and 114±9 mN/mm\textsuperscript{2} for fibers incubated with KBTBD13\textsuperscript{R408C}). However, incubation with KBTBD13\textsuperscript{R408C} significantly slowed relaxation kinetics compared to incubation with KBTBD13\textsubscript{wt} (Fig.6F, middle panel).

Together these findings suggest that in the NEM6 fibers the KBTBD13\textsuperscript{R408C} protein was not replaced by KBTBD13\textsubscript{wt} protein, but the KBTBD13\textsubscript{wt} protein was added to the endogenous KBTBD13\textsuperscript{R408C} protein. Apparently, the amount of endogenous KBTBD13\textsuperscript{R408C} protein in the NEM6 fibers was sufficient to maintain the slow relaxation kinetics. Similarly, in the healthy fibers, KBTBD13\textsubscript{wt} protein was likely not replaced by KBTBD13\textsuperscript{R408C} protein, but the KBTBD13\textsuperscript{R408C} protein was added to the endogenous KBTBD13\textsubscript{wt} protein. This addition was sufficient to exert an effect on sarcomere relaxation kinetics. These findings are in line with the R408C mutation being a dominant mutation.
Finally, it is important to note that in the experiments with permeabilized muscle fibers the cytosol is replaced by experimental solutions. Therefore, our findings suggest that KBTBD13\textsuperscript{R408C} is unlikely to act through ubiquitination or disruption of filament assembly/turnover, but rather that it regulates relaxation kinetics by direct binding to sarcomeric structures.
DISCUSSION

With the use of transcranial magnetic stimulation, we established that the origin of impaired muscle relaxation kinetics in NEM6 myopathy is myogenic. The pathomechanism underlying the observed impaired muscle relaxation kinetics was studied using contractility assays in permeabilized muscle fibers and myofibrils isolated from patient biopsies. We discovered that impaired muscle relaxation is sarcomere-based. By applying a combination of low-angle X-ray diffraction, super-resolution microscopy, modeling of muscle kinetics and proteomics with novel genetically modified mouse models and zebrafish we show that KBTBD13, the protein affected in NEM6, is an actin binding protein. Mutations in KBTBD13 slow relaxation kinetics of muscle through direct, structural effects on the actin-based thin filament. We propose that this pathomechanism is central to NEM6 pathology.

Impaired muscle relaxation in NEM6 is sarcomere-based

In vivo muscle relaxation properties were considerably slower in NEM6 patients compared to healthy controls. TMS has been used before to measure physiological muscle relaxation kinetics(25, 31–35), but this is the first time this technique has been used to assess muscle relaxation in myopathy. TMS eliminates all influences of the nervous system on muscle relaxation. Consequently, our findings demonstrate that the impaired relaxation kinetics in NEM6 patients are myogenic in nature. With a doubling of relaxation time, the muscle slowness is severe, with profound effects on mobility, as shown in the Supplementary movies. The NEM6 phenotype, i.e. impaired muscle relaxation kinetics and the absence of severe weakness (Table 1), distinguishes NEM6 from other nemaline myopathies.

The kinetics of muscle relaxation depend on the kinetics of Ca\(^{2+}\) reuptake by the sarcoplasmic reticulum and the kinetics of thick filament detachment from the thin filament. Activity levels of phospholamban, a natural inhibitor of the Ca\(^{2+}\) pumps in the sarcoplasmic reticulum, were increased in NEM6 muscle biopsies (Supplemental Fig.1F), suggesting that
slowed Ca\(^{2+}\) reuptake might contribute to the slow relaxation kinetics in muscle of NEM6 patients. In addition, the findings from histology assays suggest that the relative increase in the area of slow-twitch muscle fibers might contribute as well (slow-twitch fibers have slower contractile kinetics than fast-twitch fibers). However, slow-twitch fiber predominance is a common feature in all forms of NEM and in congenital myopathies in general whereas, clinically, impaired muscle relaxation is specific for NEM6, indicating that the increased area of slow-twitch muscle fibers is unlikely to account for the NEM6 phenotype. The findings from contractility assays in fibers and myofibrils isolated from patient biopsies, assays in which confounding effects of Ca\(^{2+}\) handling by the sarcoplasmic reticulum are absent, reveal that changes in the kinetics of thick-thin filament detachment are an important contributor to the impaired relaxation kinetics in NEM6 muscle. These changes were present in both slow- and fast-twitch fibers. Thus, impaired sarcomeric relaxation kinetics are a major contributor to slowed muscle relaxation kinetics in NEM6, irrespective of muscle fiber type.

**Thin filament structure is altered in NEM6 muscle**

Low angle X-ray diffraction studies on muscle fibers revealed that the cause of slower thick-thin filament detachment kinetics might involve structural changes of thin filaments in NEM6 muscle. The position of the actin layer line 6 reflection (ALL6), reflecting the left-handed pitch of the thin filament helix (Fig.4D), was reduced in NEM6 muscle fibers from ~5.88 to ~5.85 nm, indicating that thin filaments are more tightly wound. This compression (~0.5%, which translates to ~6 nm on a thin filament length of 1300 nm and is therefore not detectable with STED microscopy, Supplemental Fig.2F) might affect the stiffness of thin filaments. The low angle X-ray diffraction experiments in the newly-generated *Kbtbd13\(^{R408C}\)-KI* mouse model provided conclusive evidence that thin filament stiffness is increased in muscle with KBTBD13\(^{R408C}\). First, *Kbtbd13\(^{R408C}\)-KI* mice closely phenocopy NEM6 pathology, including nemaline bodies in muscle fibers and slow kinetics of muscle relaxation (Fig.5). Second, the simultaneous assessment of force generation and actin sub-unit spacing (the 2.7 nm reflection) during low angle X-ray diffraction experiments in the KI mice revealed that the
stiffness of the thin filament was increased from 37 pN*nm⁻¹*µm⁻¹ in wt muscles to 49 pN*nm⁻¹*µm⁻¹ in KI muscles (Fig.5M). The value for wt muscle is comparable to that recently reported for murine m. soleus (30 pN*nm⁻¹*µm⁻¹(27)). On the other hand, the value for KI muscle is likely an underestimation, as in the determination of myofibrillar area (required to calculate the number of individual thin filaments) we did not take into account the area occupied by nemaline rods. Figures 5C&D suggest that this area is significant. Thus, the actual stiffness of thin filaments in KI muscle might even be considerably higher than the value here reported.

To evaluate whether the increased thin filament stiffness slows muscle relaxation kinetics, we turned to simulations of sarcomere contractility kinetics, using the model of paired compliant thin and thick filaments described by Campbell(28) (Supplemental Fig.7). In these simulations, the rate of relaxation reflects the speed at which the thick filament-based myosin heads detach from actin binding sites on the thin filament. This detachment rate is accelerated by allowing myosin heads to move relative to actin. In real muscles, the relative movement between the myosin heads and actin binding sites results from, among others, thick and thin filament compliance (inverse of stiffness). In brief, during sarcomeric relaxation, the Ca²⁺ concentration near the sarcomeres falls. If a myosin head detaches under these conditions, the binding site on actin has a high probability of deactivating before another myosin head can bind. Thus, myosin heads that are detached by movement of thick filaments relative to thin filaments (due to thin and thick filament compliance, i.e. the reciprocal of stiffness) are not replaced. The prevailing force must thus be borne by a smaller number of myosin head-actin interactions. This in turn stretches the remaining interactions and accelerates their detachment. The net result is a positive feedback loop which causes thin-thick filament detachment. Our simulation results suggest that increasing thin filament stiffness, decreases the positive feedback and slows the rate of relaxation. To our knowledge, this is the first study that links increased thin filament stiffness with slow sarcomeric relaxation kinetics. The findings obtained provide insights in the mechanisms
underlying the previously reported fast relaxation kinetics of myofibrils from nebulin-deficient
mice (36); in the absence of nebulin, the thin filaments are more compliant (27).

It should be noted that the magnitude of thin filament stiffening in NEM6 muscle is unknown,
and therefore we modeled the effect of very stiff thin filaments. Hence, we cannot rule out
that in NEM6 muscle the effect of thin filament stiffness on relaxation kinetics is less
pronounced than the model predicts. Furthermore, in addition to thin filament stiffness,
increased thin filament activation in NEM6 muscle might contribute to the slow relaxation
kinetics. Interestingly, increased thin filament activation would not only slow relaxation
kinetics, but also increase the calcium sensitivity of force, as indeed observed in NEM6 fibers
(Supplemental Fig. 2D&E) and indirectly in muscle of Kbtbd13R408C-KI mice (Supplemental
Fig. 5H). We speculate that the KBTBD13R408C-induced increased flexural rigidity of thin
filaments might play a role (Fig. 6E; 30% increase in the persistence length of actin filaments
exposed to recombinant KBTBD13R408C protein). This speculation is based on previous
reports indicating that, similar to KBTBD13R408C, the actin binding protein phalloidin (1)
increases the persistence length of actin filaments (37) with (2) a concomitant increase of thin
filament activation (38). Finally, the KBTBD13-based effects on thin filament activation and on
thin filament stiffness might act in concert to modulate muscle relaxation kinetics in NEM6
muscle, a possibility that warrants further investigation.

KBTBD13 modulates thin filament function

Our data indicate that the increased stiffness of thin filaments in NEM6 muscle is a direct
consequence of binding of mutant KBTBD13 to actin. KBTBD13 is a muscle-specific protein
composed of an N-terminal BTB domain and a C-terminal Kelch-repeat domain. The role of
KBTBD13 in muscle structure and function has been largely unknown. Previous work
suggested that KBTBD13 is a substrate adaptor for Cullin-3, a muscle specific ubiquitin
ligase, and thereby implicated the ubiquitin-proteasome pathway in the pathogenesis
of KBTBD13-associated NEM (24, 39). The present study reveals that KBTBD13 has
additional functions, namely that of an actin-binding protein that modulates the relaxation
kinetics of muscle. Homology modelling suggested that KBTBD13 binds to actin (Fig.6A), which was confirmed by pull-down assays and co-sedimentation assay with filamentous actin (Fig.6B&C). APEX-based electron microscopy of GFP-tagged mutant and wt KBTBD13 in zebrafish showed localization consistent with KBTBD13 binding to actin in intact muscle fibers (Fig.6D). Of interest, the binding assay predicted a 1:8 stoichiometry of KBTBD13 to actin. This is similar to the stoichiometry of the regulatory proteins tropomyosin and troponin to actin (both 1:7). Tropomyosin, troponin, and seven actin monomers together form one regulatory unit, and a serial sequence of these regulatory units control thin filament activation and de-activation. This raises the intriguing possibility of KBTBD13 acting in concert with actin, tropomyosin, and troponin to fine-tune the kinetics of muscle contractility. It should be noted that the APEX-based electron microscopy studies in zebrafish suggest that KBTBD13 also localizes to the cytoplasm and the thick filament (Fig.6D). Indeed, the low-angle X-ray diffraction studies in Kbtbd13-KI mice showed reduced thick filament backbone periodicities (M6 and 2.8 nm reflections; Supplemental Fig.5K&L), suggesting an altered structure. Thick filament stiffness, however, was unaffected as indicated by the unaltered Δ2.8 nm reflection during muscle activation (Supplemental Fig.5M). The functional significance of KBTBD13 localization to the thick filament is unclear and warrants further investigation. The cytoplasm localization is in agreement with previous work from Sambuughin et al. in so-called ‘pre-myofibrils’ of c2c12 cells, which suggested cytosolic localization but no myofibrillar localization(19). Perhaps localization of KBTBD13 depends on the developmental stage of muscle, and doesn’t localize to the actin filament until after the pre-myofibril stage.

To date, three disease-causing mutations in KBTBD13 have been described, of which the p.R408C mutation is the most frequently described one. Here, we report two novel mutations in KBTBD13: p.I369M and p.E83Q. Notably, I369 is closely located to K390, R408 and the cysteine that might be responsible for actin-binding (C367, Fig.6A). This observation, in combination with the observed structural and functional changes in the actin-based thin filament in muscle fibers from NEM6 patients and Kbtbd13R408C-KI mice supports our
hypothesis that $KBTBD13$ mutations affect relaxation kinetics through direct effects on the thin filament. The majority of patients included in the present study have the $KBTBD13^{R408C}$ mutation (Table 1). For this reason, we generated recombinant $KBTBD13^{R408C}$ and observed that its binding affinity for filamentous actin was comparable to that of $KBTBD13^{wt}$, despite the mutation being close to C367 (Fig.6A). This suggests that the mechanism underlying the observed slowing of relaxation kinetics of human muscle fibers exposed to $KBTBD13^{R408C}$ (Fig.6F) does not involve reduced or increased binding to actin of mutant $KBTBD13$, but rather an effect on the structure of the thin filament once bound to actin. The nature of this effect is unknown. We propose that $KBTBD13^{R408C}$ is a gain-of-function mutation. This proposition is based on the observation that (1) the Kbtbd13$^{R408C}$-KI mice do not phenocopy the mice that are deficient in $KBTBD13$ (eg., unlike the Kbtbd13-KO mice, the Kbtbd13$^{R408C}$ mice have slower kinetics of muscle relaxation; Fig.5H); (2) $KBTBD13^{wt}$ protein did not affect the flexural rigidity of thin filaments, whereas $KBTBD13^{R408C}$ increased the rigidity (Fig.6E). Thus, $KBTBD13^{wt}$ protein does not affect thin filament structure and muscle relaxation kinetics, whereas $KBTBD13^{R408C}$ slows muscle relaxation kinetics by direct effects on thin filament structure.

How the putative role of $KBTBD13$ in ubiquitin ligase activity is involved in these effects on muscle relaxation kinetics is unknown. An important aspect of the experimental approach was that in the assays in which the effect of $KBTBD13$ on relaxation was studied, permeabilized muscle fibers were used (Fig.6F). In permeabilized muscle fibers the cytosol is replaced by an experimental solution in which no ubiquitin and conjugation enzymes are present. Thus, in this context the modulation of relaxation kinetics by $KBTBD13^{R408C}$ protein (Fig.6F) must be a consequence of direct binding to sarcomeric proteins, presumably actin, rather than of ubiquitin ligase or other cytosolic activity. Note that the newly identified mutation E83Q is located in the BTB domain of $KBTBD13$, whereas the other four mutations are located in the Kelch domain. The BTB-domain interacts with Cul3, whereas the Kelch domain provides an adaptor function for substrate recognition(24, 39). Based on our findings, we postulate that actin is targeted by $KBTBD13$'s Kelch domain, and we speculate that the
mutation in the BTB domain may affect sarcomere function through changes in ubiquitination.

In conclusion, this study identified KBTBD13 as an actin-binding protein, a property that when affected by mutations alters the structure of the thin filament thereby impairing muscle relaxation kinetics. We propose that this pathomechanism is central to the NEM6 phenotype.
METHODS

Please see the Supplemental Methods for a detailed description of: transcranial magnetic stimulation, muscle biopsies, permeabilized muscle fiber mechanics, myosin heavy chain isoform composition determination, myofibril mechanics, recombinant KBTBD13 and KBTBD13\textsuperscript{R408C} protein expression, actin and KBTBD13/KBTBD13\textsuperscript{R408C} co-sedimentation assay, pull-down assay with recombinant KBTBD13 proteins, mass spectrometry, KBTBD13 and KBTBD13\textsuperscript{R408C} incubation assay in human muscle fibers, Generation of the \textit{Kbtbd13}\textsuperscript{-} knock out and \textit{Kbtbd13}\textsuperscript{R408C}-knock in mouse models, Intact muscle mechanics, homology modeling, KBTBD13 localization in zebrafish, antibodies used for Western blot, flexural rigidity of actin filaments, electron microscopy, immunohistochemistry, Ca\textsuperscript{2+} re-uptake, low-angle X-ray diffraction and Stimulated Emission-Depletion Microscopy.

Statistics

Data are presented as mean ± standard error of the mean or as median with interquartile range (normally distributed, not normally distributed, respectively). For statistical analyses, two-tailed \textit{t} tests or Mann-Whitney U tests were used. A probability value < 0.05 was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

Study approval

Experimental procedures of human studies were approved by the institutional ethics committee and performed in accordance with ethical standards laid down in the Declaration of Helsinki. All biopsies were collected following informed consent supervised by the Radboud University Institutional Review Board. All murine experiments were approved and conducted under the supervision of the University of Arizona IACUC and followed the NIH Guidelines 'Using Animals in Intramural Research'.

AUTHOR CONTRIBUTION
Designing research studies: JMdW, CACO, DR, BB, RB, RJBR, TI

Conducting experiments: JMdW, JPM, MY, RvdP, SS, SC, MvdL, MW, SJPB, EvK, SL, MP, TES, AAR, VO, GR, TEH, ZX, CNJ, FL, BK, NLV, RB, MM, LN, RJB, BK, JD, KC, WM, EM

Acquiring data: JMdW, JPM, MY, Rvd, SS, SC, MvdL, MW, SJPB, EvK, SL, MP, TES, AAR, VO, GR, TEH, ZX, CNJ, FL, BK, NLV, RB, MM, LN, RJB, BK, JD, KC, WM, EM

Analyzing data: JMdW, JPM, MY, Rvd, SS, SC, MvdL, MW, SJPB, EvK, SL, MP, TES, AAR, VO, GR, TEH, ZX, CNJ, FL, BK, NLV, RB, MM, LN, RJB, BK, JD, KC, WM, EM

Writing the manuscript. JMdW, JPM, CACO, NV, BGMvE, HG, AHB, NR

Justification order of shared first authorship: Both JMdW and JPM provided the majority of work in this study: JMdW took responsibility for the pre-clinical part and JPM for the clinical work. As during the study progress, the pre-clinical part has increased significantly over the clinical work, JMdW is now listed as first author and JPM as second.
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18. Gupta VA et al. Identification of KLHL41 Mutations Implicates BTB-Kelch-Mediated


Supplemental Figure 1. Calcium handling protein and activity levels.

(A) Representative western blot of quadriceps homogenates from NEM6 patients and control subjects (CTRL) probed for key proteins involved in sarcoplasmic reticulum Ca^{2+} handling. (B) No differences were observed in SERCA1 and SERCA2 protein levels (normalized to actin) between samples from CTRL and NEM6 patients. (C) SERCA1 protein levels correlate with the area of fast-twitch in muscle biopsies of NEM6 patients. (D) SERCA1 activity levels of sarcoplasmic reticulum vesicles isolated from NEM6 muscles are lower than those of control subjects. (E) SERCA1 activity levels correlate with the area of fast-twitch fibers in muscle biopsies of NEM6 patients. (F) The ratio of phospholamban (active form) over phosphorylated phospholamban (inactive form) is increased in NEM6 muscle biopsies. T-tests were performed between NEM6 and CTRL; \( P < 0.05 \) was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

Supplemental Figure 2. Fiber area and contractility in NEM6 patients.

(A) The cross-sectional area of slow-twitch and fast-twitch fibers from control subjects (CTRL) that were used for mechanics was of similar size; in NEM6 biopsies, the cross-sectional area of slow-twitch fibers was increased compared to fast-twitch fibers. (B) The fiber size distribution (type 1 are slow-twitch fibers; type 2A/2B/2X are fast-twitch fibers) in muscle biopsies of CTRL and NEM6 patients as determined by histology assays. (C) Slow-twitch (type 1) fiber diameter relative to fast-twitch (type 2A/2B/2X combined) fiber diameter is increased in NEM6 patients, as determined by histology assays. (D) In slow twitch fibers, (left panel) the absolute force (determined at incremental \([\text{Ca}^{2+}]\)) is comparable in NEM6 patients and control subjects (CTRL), and (right panel) the calcium sensitivity of force is higher in NEM6 patients compared to CTRL as reflected by the higher pCa_{50} (inset). (E) In fast-twitch fibers, (left panel) the absolute force is lower in NEM6 patients compared to CTRL, and (right panel) the calcium sensitivity of force is higher in NEM6 patients compared to CTRL as reflected by the higher pCa_{50} (inset). (F) Upper left panel: The sarcomere length
dependence of maximal tension (at pCa 4.5) was not different between NEM6 and control fibers (CTRL). **Upper right panel**: No changes in thin filament length were observed between NEM6 and CTRL fibers. **Middle panel**: The actin-based thin filament in CTRL fibers was stained with phalloidin and imaged by super-resolution STED microscopy. Thin filament length was analyzed by performing line scans along the length of the fiber. The half width at half maximum intensity of a Gaussian fit with plateau phase was used to indicate thin filament length. Lower panel: STED microscopy image and line scan of a NEM6 fiber. T-tests were performed; p < 0.05 was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

**Supplemental Figure 3. Passive stiffness of permeabilized muscle fibers.**

Typical example of (A) the applied changes in length of a control muscle fiber (CTRL) and (B) the development of passive tension in time. (C) No differences in passive tension were observed at incremental sarcomere lengths between muscle fibers of control subjects and NEM6 patients. (D) The passive tension measured at the end of the hold phase, at a sarcomere length of 3.2 µm, was not different between NEM6 patients and control subjects. T-tests or Mann-Whitney U-tests were performed between NEM6 and CTRL; P < 0.05 was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

**Supplemental Figure 4. Nemaline rods.**

Electron microscopy image of muscle of a NEM6 patient, highlighting the presence of nemaline rods (example indicated by white arrow).

**Supplemental Figure 5. Characteristics of the Kbtbd13R408C mouse model.**

(A) Homozygous KI mice are born at higher than Mendelian ratios (KI: 19%; wt 31%; HET: 50%). (B) PCR result, showing the wt, HET and homozygous (KI) products. (C) Expression of mutant (KBTBD13R408C) mRNA in m. soleus of wt, heterozygous (HET) and homozygous
(KI) mice. (D) Muscle weights in nine month old KI and wt mice. (E) Left: Picro-sirius stained
cryosections of m.soleus (scale bar = 200 µm). Right: Cryosections of m. soleus stained with
antibodies against type 1 (green) and type 2A (blue) myosin heavy chain isoforms (scale bar
= 100 µm). (F) CSA of both slow-twitch (ST) and fast-twitch (FT) fibers of m. soleus is larger
in KI than in wt mice; the difference is more pronounced in ST fibers (*: different between wt
and KI; #: different between ST and FT). (G) Soleus muscle of KI mice shows preferential
hypertrophy of slow-twitch fibers. (H) The force-frequency relation of m. soleus of wt and KI
mice. The relation is shifted leftwards in KI muscle. (I) Single twitch half-relaxation time in m.
soleus of KI mice is longer than in muscle of wt mice. (J) SERCA1 levels are not significantly
different in m. soleus between wt and KI mice. The spacings of the (K) M6-reflection and (L)
the 2.8 nm reflections (both associated with the thick filament backbone periodicity) are
reduced in KI mice. (M) The change in the spacing of 2.8nm-reflection (∆2.8 nm) during
maximal tetanic activation is comparable between KI and wt mice, indicating comparable
thick filament extension. T-tests were performed between KI and wt mice; P < 0.05 was
considered statistically significant. For detailed information on the number of samples and
statistical tests and outcomes, please see Supplemental Table 1.

Supplemental Figure 6. Characteristics of the Kbtbd13 KO mouse model.
(A) Left: Photo of a Kbtbd13-KO and wt mouse; Right: PCR result showing the primer
products of wt, (HET) and KO mice. PCR result, showing the wt, heterozygous (HET) and
KO products. (B) KO mice are born at close to Mendelian ratio (KO: 21%; wt 28%; HET:
50%). (C) Expression of Kbtbd13 mRNA in severely reduced in KO mice. (D) Body weight of
9 month old KO mice is lower than that of wt mice. (E) Muscle weights in 9 month old KO and
wt mice. (F) Maximal tetanic tension (150Hz) is lower in m. soleus of KO mice than of wt
mice. (G) The force-frequency relation of m. soleus of 9 month old wt and KO mice. The
relation is comparable between KO and wt mice. (H) SERCA1 levels are not significantly
different in m. soleus between wt and KO mice. (I) Single twitch (left panel) and maximal
tetanic (right panel) half-relaxation times in m. soleus are comparable between wt and KO
mice. T-tests were performed between KO and wt mice; $P < 0.05$ was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

**Supplemental Figure 7. Schematic of sarcomere modelling**

(A). Schematic showing the model of compliant filaments. (Modified from Fig. 5 of Campbell 2016). Each thick filament was modeled as a chain of 18 myosin heads joined by linear springs of stiffness $k_m$. Thin filaments were composed of 189 binding sites organized into 27 regulatory units; each binding site was joined to its neighbors by linear springs of stiffness $k_a$. The force in a single cross-bridge link was defined as $k_{cb} (x + x_{ps})$, where $k_{cb}$ is the stiffness of the link, $x$ is the cross-bridge displacement, and $x_{ps}$ is the cross-bridge power stroke. All of the sites in a regulatory unit switched between the available and unavailable states at the same time. Sites could not switch to the unavailable state if one of the sites in the regulatory unit was bound by a myosin head. For the present simulations, the stiffness of the actin filament was increased by a factor of 1000. (B) Simulation modeling of compliant and stiffened thin filament dynamics. **Top left:** Example shows activation pattern of simulation modeling of compliant and stiffened thin filament dynamics; **Top right:** maximal force was not affected by stiffening of the thin filament (SD based on 500 simulations); **Bottom right:** relaxation kinetics were slower in the model with stiffer thin filaments.

**Supplemental Figure 8. Transgenic Zebrafish.**

(A) Confocal microscopy images of transgenic KBTBD13<sup>wt</sup>-GFP zebrafish and (B) transgenic KBTBD13<sup>R408C</sup>-GFP zebrafish, co-stained for sarcomeric alpha-actinin (red) with the corresponding intensity profiles. Note that due to chromatic aberrations caused by the difference in wavelength of the fluorophores, the fluorescence in the images might appear slightly shifted (scale bar = 5 µm and 2 µm (zoom)). (C) Note that this image is also shown in figure 6D (top, left). APEX-based electron microscopy of zebrafish expressing GFP-tagged
KBTBD13<sup>wt</sup> or GFP-tagged KBTBD13<sup>R408C</sup> protein. Fish had mosaic expression, with positive (indicated with '+') and negative ('-') cells. Positive cells stain dark in electron micrographs. Note that in both the fish expressing wt and mutant protein, the myofibrils show dark staining (scale bar = 5 µm).

**Supplemental Figure 9. Flexural rigidity.**

Note that this image is also shown in Fig. 6E. Representative image of actin filaments in solution. Inset illustrates the determination of the parameters required to calculate the persistence length (Lp) of the actin filaments. The equation used to calculate Lp is shown left to the image.

**Supplemental Videos.**

**Clinical phenotype of a NEM6 patient.**

On neurological examination the patient displayed generalized muscle weakness. MRC: neck flexion 3, neck extension 4, shoulder girdle 4; positive Trendelenburg while walking, walking upstairs and running.

**Video 1:** Walking stairs. The patient walks up and down the stairs: here, at voluntary speed. Note that she needs the support of both hands to walk upstairs.

**Video 2:** Walking stairs. The patient walks up and down the stairs: here, as fast as she can. When walking up the stairs rapidly, she experiences muscle slowness in addition to weakness: she reports a feeling that her legs cannot keep up with her.

**Video 3:** Walking and running. The patient walks down the hall. When returning the patients is asked to run as fast as she can. During running, she experiences muscle slowness in addition to weakness: she reports a feeling that her legs cannot keep up with her.
Flexural rigidity assessment of actin filaments in solution.

Video 4: Actin filaments in solution, no protein added. Ten frames per sec.

Video 5: Actin filaments in solution, KBTBD13<sup>wt</sup> added. Ten frames per sec.

Video 6: Actin filaments in solution, KBTBD13<sup>R408C</sup> added. Ten frames per sec.
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All the biopsies that showed cores and/or rods also showed myopathic changes including an increase of internal nuclei, fiber type disproportion, and fiber type I predominance.

NA  Not assessed
*  Muscle weakness: Proximal muscle groups are more affected than distal ones. Axial weakness is also present. No facial weakness.
** Mild: Strength is at least MRC 4
*** Moderate: Strength is MRC 3-4
#  This patient has predominantly distal weakness and thus differs from the other patients
Figure 1

A. Muscle fiber
Myofibril
Sarcomere
Thin filament
Thick filament

B. Myosin 18B
Myopalladin
Cofilin-2
Actin
α/β-Tropomyosin
Troponin complex
KBTBD13
KLHL41
KLHL40
Nebulin
Leiomodin-3

~100 μm
~1 μm
~23 nm
~23 nm
Figure 1. Schematic of muscle from the macroscopic to the nanoscopic level.

(A) Simplified schematic of a skeletal muscle fiber, myofibril, sarcomere, thick and thin filament: key components of muscle activation and relaxation. (B) Simplified schematic of the skeletal muscle thin filament. The majority of genes implicated in nemaline myopathy encode thin filament (associated) proteins. For KBTBD13, the localization and function are unknown. Arrows indicate direct associations, dotted arrows indicate indirect associations.
Figure 2

A. Motor cortex stimulation
Finger flexor EMG
Handgrip dynamometer

B. Force (%max)

Female
Male

C. Force (N)

CTRL NEM6
CTRL NEM6

D. Normalized pRR (s⁻¹)

CTRL NEM6

0.75 Relaxation Time (ms)

CTRL NEM6
Figure 2. Transcranial Magnetic Stimulation to study *in vivo* muscle relaxation kinetics.

(A) Schematic of the experimental setup for Transcranial Magnetic Stimulation (TMS) to induce involuntary muscle relaxation (top). Typical, superimposed, force traces and corresponding electromyogram (EMG) of a control subject (CTRL) and NEM6 patient during maximal voluntary contraction and TMS-induced involuntary relaxation of the deep finger flexors (middle and bottom). Note the motor-evoked potential on the EMG traces corresponding to the small superimposed twitch, and the EMG silent period of approximately 200 ms corresponding to the drop in force after which voluntary force is generated again. Note the slower relaxation in the NEM6 patient.

(B) Maximal force is lower in NEM6 patients than in CTRL, both in males and in females. (C) The peak relaxation rate (normalized to maximal force; pRR) is lower in NEM6 patients than in controls. (D) The time to 75% of maximal force (0.75 relaxation time) is longer in NEM6 patients than in controls. Orange circle corresponds to the NEM6 patient harboring the *KBTBD13*<sup>K390N</sup> mutation; open circles to those with the *KBTBD13*<sup>R408C</sup> mutation. T-tests were performed between NEM6 and CTRL; *P* < 0.05 was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.
Figure 3
Figure 3. Contractility assays to study relaxation kinetics of muscle fibers and myofibrils.

(A) Electron microscopy images from muscle fibers of a control (CTRL) and (B) a NEM6 patient (note the areas with myofibrillar damage, indicated by asterisk). (C) Light microscopy images of a slow-twitch and (D) a fast-twitch NEM6 patient fiber that were used for contractility assays. (E) Maximal tension of both slow-twitch and (F) fast-twitch fibers from NEM6 patients was significantly lower than that of CTRL. (G) Typical relaxation trace of a NEM6 and a CTRL muscle fiber. (H) The kinetics of relaxation after maximal activation – reflected by the relaxation constant $K_{rel}$ – were markedly slower in both slow-twitch and fast-twitch fibers of NEM6 patients when compared to CTRL. The orange, green and yellow circles correspond to NEM6 patients harboring the $KBTBD13^{K390N}$, $KBTBD13^{I369M}$ and $KBTBD13^{I369M}$, respectively. The open circles correspond to patients with the $KBTBD13^{R408C}$ mutation. (I) Electron microscopy images indicate a myofibril (highlighted in white) from muscle fibers of CTRL and (J) a NEM6 patient. Note that these EM images are higher magnifications of the same images in Fig. 3A and Fig. 3B, respectively. (K) Myofibril mounted between a force probe and length controller for contractility assays. (L) The maximal tension of myofibrils of NEM6 patients was lower than that of CTRL. (M) Typical example of the activating kinetics and (N) the relaxation kinetics of a NEM6 and a CTRL myofibril; note that activation and relaxation kinetics are from the same myofibril. (O) The relaxation kinetics were slower in myofibrils of NEM6 patients than in those of CTRL. $T$-tests or Mann-Whitney U-tests were performed between NEM6 and CTRL; $P < 0.05$ was considered statistically significant. For each biopsy, four to twelve muscle fibers/myofibrils were measured. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.
Figure 4

A. X-ray beam

B. Sarcomere

C. M6 peak position (nm)

D. ALL6 peak position (nm)

E.

F.
Figure 4. Determination of the nanostructure of muscle fibers.

(A, top) Picture of twenty-eight NEM6 muscle fibers mounted and aligned in one plane between two-halves of an electron microscopy (EM) grid; (bottom) typical example of the resulting low angle X-ray diffraction pattern. Note the well resolved equatorial and meridional reflections. (B, left) Schematic of a sarcomere and the thin and thick filament, highlighting the structures that underlie the actin layer line 6 (ALL6) reflection and the myosin (M6) reflection; (right) schematic representation of a cross-section of muscle (thin filament in yellow, thick filament in pink), indicating the 1,0 and the 1,1 reflections. (C) No change in M6-reflection (thick filament backbone periodicity) is observed in fibers of NEM6 patients compared to those of controls. (D) The actin layer line 6 reflection (ALL6) is reduced in NEM6 fibers compared to control fibers. The green circles and open circles correspond to NEM6 patients harboring the KBTBD13<sup>369M</sup> mutation and the KBTBD13<sup>408C</sup> mutation, respectively. T-tests or Mann-Whitney U-tests were performed between NEM6 and CTRL; $P < 0.05$ was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.
Figure 5

A. KI vs. WT

B. Graph showing body weight (g) distribution for WT and KI.

C. Images of WT and KI tissues.

D. Fluorescence microscopy image.

E. Phal intensity (A.U.) over distance (μm) for WT and KI.

F. MHC intensity (A.U.) over distance (μm) for WT and KI.

G. Maximal tetanic tension (mN/mm²) vs. sarcomere length (μm) for WT and KI.

H. 1/2 relaxation time (s) for WT and KI.

I. Diagram showing thin filament length (nm) with 2.7nm and 2.8nm.

J. ALL6 peak position (nm) forWT and KI.

K. 2.7 nm spacing for WT and KI.

L. Δ27 nm in % for WT and KI.

M. Thin filament stiffness (pN nm⁻¹ μm⁻¹) for WT and KI.
Figure 5. Characteristics of the *Kbtbd13*R408C KI mouse model.

(A) Image of a *Kbtbd13*R408C wt and homozygous KI mouse. (B) Body weight was significantly higher in KI mice. (C) Gomöri trichrome staining of m. soleus cryosections (scale bar = 100 µm). KI section shows nemaline rods in many muscle fibers (examples indicated by white arrow). (D) Phalloidin staining of a permeabilized fiber of m. soleus; note the abundance of actin-positive nemaline rods. (E/F) STED microscopy images (deconvolved) of permeabilized fibers of m. soleus stained with phalloidin (PHAL; to stain the thin filament) and with a pan-specific myosin heavy chain antibody (MHC; to stain the thick filament); right panels show the resulting thin and thick filament lengths as a function of sarcomere length. (G) Maximal tetanic tension (150Hz) of m. soleus was comparable between wt and KI mice. (H) The maximal tetanic half-relaxation time was significantly longer in m. soleus of KI than in wt mice. (I) Typical example of a low angle X-ray diffraction pattern obtained from m. soleus of a *Kbtbd13*-KI mouse; note the well resolved reflection of the actin sub-unit spacing (2.7nm). (J) The spacing of the actin layer line 6 reflection (ALL6) is reduced in muscle of *Kbtbd13*R408C-KI mice. (K) the actin sub-unit spacing (2.7 nm) is reduced in muscle of *Kbtbd13*R408C-KI mice. (L) The change in the actin sub-unit spacing (\(\Delta 2.7 \text{ nm}\)) during maximal tetanic activation is smaller in *Kbtbd13*R408C-KI mice, indicating less thin filament extension. (M) Thin filament stiffness is higher in muscle of *Kbtbd13*R408C-KI mice than in muscle of wt mice. T-tests were performed between KI and wt mice; \(P < 0.05\) was considered statistically significant. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.
Figure 6

A. Beta propeller from KBTBD13

B. GST-

C. KBTBD13-WT

D. Serial dilution

E. Actin filament

F. KBTBD13-WT

Figure 6
Figure 6. Effect of KBTBD13 on sarcomere structure and function.

(A) Homology modelling reveals that the KBTBD13 beta propeller motifs (left panel) have striking similarities with those of the actin binding protein scruin in limulus sperm (middle panel). KBTBD13 has a cysteine residue (Cys367) located in nearly the exact same position as scruin (Cys837, right panel). The KBTBD13R408C, KBTBD13I369M, and KBTBD13K390N mutations are in close proximity to Cys367. (B) SDS-PAGE image after a pulldown assay in a mouse muscle lysate (muscle lys.) with GST only (left lane), GST-KBTBD13 (second lane from left, GST-KBTBD13 without lysate ... band (*) was identified, excised, and analyzed with mass-spectrometry. Actin was prominently present in the excised band. (C, left panel) The amount of KBTBD13 (gray) and KBTBD13R408C (blue) bound to actin (corrected for sedimentation without actin) versus free KBTBD13 were plotted and fitted to the Hill equation. Individual fits for each protein were compared with a global fit using Akaike’s Information Criteria and a shared curve was found to adequately fit both datasets suggesting recombinant KBTBD13 and KBTBD13R408C bind to filamentous actin with comparable affinities. The shared fit and its 95% confidence interval are shown in black. (C, right panel) Representative Western blot of KBTBD13 in the pellet and supernatant fraction (in the presence of actin, P+ and S+, respectively) and the pellet fraction in the absence of actin (P-) (lane 1-3). Note, that the amount of polymeric actin (as determined by the amount of actin in the pellet fraction) is not different in the presence or absence of KBTBD13 (lane 1 and lane 4). Lane 6-8 show dose-dependent detection of a serial dilution of recombinant KBTBD13. (D) APEX-based electron microscopy of zebrafish expressing GFP-tagged KBTBD13. Top left: Fish had mosaic expression, with positive (indicated with ‘+’) and negative (‘-’) cells. Positive cells stain dark in electron micrographs. Note that in the cells, the myofibrils in particular show dark staining (scale bar = 1 µm). Top right: To increase the contrast between KBTBD13-positive and -negative areas, the images were converted from gray scale to spectrum scale, with KBTBD13 in light blue. Below, right: high magnification of the myofibrillar compartment (scale bar = 0.3 µm). Within the myofibrils, the A-band (mainly myosin-based thick filaments) and the I-band (mainly actin-based thin filaments) show labeling, indicating that KBTBD13 binds to the thin filament and the thick filament. Furthermore, the z-discs, where thin filaments from adjacent sarcomeres overlap, show strong labeling. Below, left: high magnification of the myofibrillar compartment of a negative cell (scale bar = 0.3 µm). A-band and I-band do no show labeling. (E) Left panel: representative image of actin filaments in solution. Inset illustrates the determination of the parameters required to calculate the persistence length (Lp) of the actin filaments. Right panel: KBTBD13wt protein does not affect Lp, but KBTBD13R408C increases Lp of actin filaments (more details on the Lp calculations are in Supplemental Fig.9; Videos of the actin filaments in solution are shown in Supplemental videos 4-6). (F). Left panel: Typical example of relaxation traces of two segments of the same permeabilized muscle fiber: one incubated with recombinant KBTBD13wt, the other with recombinant KBTBD13R408C. Right panel: In healthy control muscle (CTRL), incubation with KBTBD13R408C slows relaxation kinetics of permeabilized muscle fiber segments compared to that of the segments incubated with KBTBD13; In NEM6 muscle, incubation with KBTBD13wt does not affect the relaxation kinetics of permeabilized muscle fiber segments compared to that of the segments incubated with KBTBD13R408C. T-tests or Mann-Whitney U-tests were performed between For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.
Figure 7. Graphic summary of the proposed pathomechanism in NEM6.