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Graphical abstract

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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 superresolution 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 and binding and contractility assays with recombinant KBTBD13, Kbtbd13-knockout and Kbtbd13<sup>G448R</sup>-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 (Figure 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 Supple-
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The 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 (TMS-induced) in vivo muscle-relaxation studies, in vitro muscle fiber- and sarcomere-contractility assays, and low-angle x-ray diffraction and superresolution 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, Kbtbd13-KO and Kbtbd13R408C-knockin (Kbtbd13R408C-KI) 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 (a) KBTBD13 as a regulator of skeletal muscle relaxation and (b) the pathomechanism 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 noninvasive TMS (25) (Figure 2A). Maximal force was lower in NEM6 patients than in control subjects, both in males and in females (Figure 2B). Because relaxation kinetics were similar between males and females, these data were pooled. Figure 2A 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 (Figure 2C), and accordingly, the 75% relaxation time (time for force to drop by 25%) was longer in NEM6 patients than in controls (Figure 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 Ca2+ handling proteins contributed to impaired muscle relaxation in NEM6. The level of SERCA1, a key protein involved in sarcoplasmic reticulum Ca2+ handling in fast-twitch fibers, was not significantly different between NEM6 and control muscle (Supplemental Figure 1, A and B). SERCA1 protein levels and SERCA activity strongly correlated with 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.

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As phospholamban slows SERCA activity, this might contribute to slower muscle relaxation in NEM6. Electron microscopy (EM) analyses showed no aberrations in the structure of the triads in muscle fibers of NEM6 patients.

The area of fast-twitch fibers in muscle biopsies of NEM6 patients (Supplemental Figure 1, C–E). The ratio of phospholamban (active form) over phosphorylated phospholamban (inactive form) was increased in NEM6 muscle biopsies (Supplemental Figure 1F).

### Table 1. Clinical characteristics and genetic information of patients and controls

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<th>Reported muscle slowness/stiffness</th>
<th>Result of biopsy</th>
<th>KBTD13 mutation</th>
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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. aMuscle weakness: proximal muscle groups are more affected than distal ones. Axial weakness is also present. No facial weakness. bMild: strength is at least MRC scale 4. cModerate: strength is MRC scale 3–4. dThis patient has predominantly distal weakness and thus differs from the other 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\textsuperscript{2+}. Patient characteristics are shown in Table 1. Figure 3, A and B, shows EM images of muscle fibers from a control and an NEM6 patient, and Figure 3, C and 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 Figure 2A), indicating that the fibers used for mechanics reflect the fiber size distribution in the whole biopsy as determined by histology (Supplemental Figure 2, B and 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 with those of control subjects, but not in slow-twitch NEM6 fibers compared with those of controls (Supplemental Figure 2, D and E). 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 approximately 50%; Figure 3, E and 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 Figure 2F). This finding was in line with the unaltered thin-filament length in NEM6 fibers, as determined by superresolution-stimulated emission depletion (STED) microscopy (Supplemental Figure 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 Figure 2, D and E). Note that we observed no changes in the passive stiffness of NEM6 muscle fibers (Supplemental Figure 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 with control subjects. Figure 3G shows a typical relaxation trace of a patient’s and a control subject’s muscle fibers. The relaxation kinetics, reflected by the relaxation constant $K_{rel}$, were approximately 3-fold slower in NEM6 fibers (Figure 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; Figure 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 with control fibers (Supplemental Figure 2, A–C), which affects diffusion time of solutions. Therefore, we also measured the contractility of...
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 Figure 3M), the relaxation kinetics were slower in myofibrils of NEM6 patients than in those of controls. Student’s t tests or Mann-Whitney U tests were performed between NEM6 and controls. *P < 0.05. For each biopsy, 4 to 12 muscle fibers/myofibrils were measured. For detailed information on the number of samples and statistical tests and outcomes, please see Supplemental Table 1.

NEM6 myofibrils. Figure 3, A B, I, and J, shows EM images of a control and an NEM6 muscle fiber (1 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 1 muscle fiber (note that nemaline rods were frequently observed in EM images of the NEM6 patients; for a high-magnification example, see Supplemental Figure 4). We isolated single myofibrils from the intact areas of NEM6 muscle fibers and measured their contractile function (experimental arrangement shown in Figure 3K). As shown in Figure 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 Figure 3M), the relaxation kinetics were slower in myofibrils of NEM6 patients than in those of control subjects (Figure 3, N and O; note that Figure 3N shows the same myofibril as in Figure 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 toward 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 28 fibers were mounted and aligned in 1 plane between 2 halves of EM grids. This approach allowed for high-quality equatorial and meridional diffraction patterns (for example, see Figure 4A). The spacing of the equatorial reflections indicated that lattice spacing was not different between NEM6 and control fibers (distance between thick filament planes (d1,0); see Figure 4B; 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/I1,0, where I1,1 indicates intensity resulting from 1,1 planes; see Figure 4B); 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 (M3 reflection: 14.53 ± 0.02 nm for control and 14.46 ± 0.03 nm for NEM6 fibers) and the thick-filament backbone periodicity (M6 reflection, Figure 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 (Figure 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 Kbtbd13R408C mice

To further investigate the effect of the Dutch founder mutation KBTBD13R408C on muscle-relaxation kinetics and thin-filament stiffness, we engineered a Kbtbd13 R408C-KI mouse model (Figure 5A). Mice were studied at 9 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 Figure 5A). Quantitative PCR showed that homozygous KI mice expressed only mutant Kbtbd13 and that heterozygous mice expressed approximately 50% of mutant and 50% of WT Kbtbd13 (Supplemental Figure 5C). KI mice had higher body weight than WT mice (Figure 5B). Soleus and diaphragm muscle weights were higher in KI compared with WT mice (Supplemental Figure 5D). Picrosirius red–stained cryosections of m. soleus (representative images in Supplemental Figure 5E) showed a significantly increased area of extracellular matrix in KI compared with WT mice (14% ± 1% vs. 12% ± 1%, respectively). Soleus muscle of KI mice had a lower percentage of slow-twitch (type 1) fibers than that of WT mice (28% ± 2% vs. 60% ± 3%, respectively; Supplemental Figure 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 Figure 5F). Similarly to what occurs in NEM6 patients (Supplemental Fig-
In KI mice, the cross-sectional area of slow-twitch fibers was larger than that of fast-twitch fibers (Supplemental Figure 5G). Gomori trichrome–stained sections of m. soleus showed nemaline bodies in muscle fibers (Figure 5C), a key characteristic of NEM. This finding was confirmed in fibers stained with phalloidin, showing actin-positive nemaline bodies (Figure 5D). Thin-filament and thick-filament length in muscle of KI mice was comparable to that in WT mice, as determined by superresolution STED microscopy (Figure 5, E and F). Thus, the pathology observed in Kbtbd13-KI mice resembled NEM pathology.

**Relaxation kinetics.** Maximal tetanic tension (150 Hz) of m. soleus was comparable between KI and WT mice (Figure 5G). The maximal tetanic half-relaxation time was significantly longer in m. soleus of KI than in WT mice (Figure 5H). 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 subunit spacing (2.7 nm) (Figure 5I). The change in the actin subunit spacing (ΔA2.7 nm) during maximal tetanic activation is smaller in Kbtbd13-KI mice, indicating less thin-filament extension (Figure 5J). Thin-filament stiffness is higher in muscle of Kbtbd13-KI mice than in muscle of WT mice (Figure 5M). Student’s t tests were performed between KI and WT mice. *P < 0.05. For detailed information on the number of samples and statistical tests and outcomes, see Supplemental Table 1.
Normal relaxation kinetics in muscle of Kbtbd13-KO mice

We also generated a Kbtbd13-KO mouse model to study the effect of absence of Kbtbd13 on muscle-relaxation kinetics. Similarly to the mice of the Kbtbd13R408C KI model, mice were studied 9 months after birth.

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

Relaxation kinetics. The maximal tetanic tension (150 Hz) of m. soleus was reduced in KO mice (Supplemental Figure 6F), and the force-frequency relation was comparable between KO and WT mice (Supplemental Figure 6G). The level of SERCA1 was not significantly different in KO compared with WT muscle (Supplemental Figure 6H). Importantly, similarly to observations in NEM6 mice, the relaxation kinetics of both single twitches and maximal tetani were comparable between KO and WT mice (Supplemental Figure 6I). Thus, the relaxation kinetics of muscle of Kbtbd13-KO mice do not mimic those of Kbtbd13R408C-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 Figure 7A). Increasing the stiffness of the actin filaments slowed the rate of relaxation from 2.53 s⁻¹ to 4.37 s⁻¹ (Supplemental Figure 7B) without producing a substantial change in maximum isometric force (Supplemental Figure 7B). 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 KBTBD13 increase thin-filament stiffness and that increased thin-filament stiffness can slow sarcomeric relaxation kinetics.

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%), 3 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 3 resulting models (root mean square deviation [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 \( \alpha \)-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. Different templates were used for constructing the scruin homology model to avoid bias. We found several similarities between the \( \beta \) propeller motif of KBTBD13 and scruin (Figure 6A). In both \( \beta \) propeller motifs,
the majority of cysteines were contained internally within the β sheets. In scrin, a single cysteine (Cys837) located in a loop at the surface of the β 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 scrin Cys837 (Figure 6A). Scrin is known to bind to filamentous actin, and Cys367 is important for this binding. These findings suggest that KBTBD13 might interact with actin. Strikingly, the location of 3 KBTBD13 mutations (I569M, K390N and R408C) are in close proximity to Cys367 (Figure 6A).

To test whether KBTBD13 binds to actin, we performed pull-down assays with full-length KBTBD13 and muscle lysates. The pulled down proteins were separated on an SDS-PAGE gel (Figure 6B). One unique band was observed at a height that corresponds to a molecular weight of 40–45 kD. 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 cosedimentation assay with filamentous actin. The data demonstrate that both KBTBD13 and KBTBD13R408C bind to filamentous actin with comparable affinities (Figure 6C). 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 approximately 1.08 ± 1.92 μM KBTBD13 to 8 μM actin, suggesting a stoichiometry of approximately 1 KBTBD13 molecule to 8 actin molecules. Recombinant KBTBD13 also showed some sedimentation in the absence of actin (Figure 6C, 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. Glutathione S-transferase (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 2 custom-made antibodies). Therefore, we generated transgenic Kbtbd13-EGFP and Kbtbd13R408C-EGFP zebrafish. Note that in these fish, the expression of Kbtbd13R408C was driven by the endogenous Kbtbd13 promoter, but the KBTBD13 WT protein was added to the endogenous KBTBD13R408C protein. Apparently, the amount of endogenous KBTBD13R408C protein in the NEM6 fibers was sufficient to maintain the slow-relaxation kinetics. Similarly, in the healthy fibers, KBTBD13 WT protein was likely not replaced by KBTBD13R408C protein, but the KBTBD13R408C protein was added to the endogenous KBTBD13 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 KBTBD13R408C 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 TMS, 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, superresolution microscopy, modeling of muscle kinetics, and proteomics with genetically modified mouse models and zebrafish, we showed that KBTBD13, the protein affected in NEM6, is an actin-binding protein. Mutations in KBTBD13 slowed 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 with healthy controls. TMS has been used before to measure physiological muscle-relaxation kinetics (25, 31–35), but this is the first time, to our knowledge, that 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 was severe, with profound effects on mobility, as shown in the Supplemental Videos. 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 Figure 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, revealed 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 ALL6, reflecting the left-handed pitch of the thin-filament helix (Figure 4D), was reduced in NEM6 muscle fibers from approximately 5.88 to approximately 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 Figure 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 (Figure 5). Second, the simultaneous assessment of force generation and actin subunit 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 (Figure 5M). The value for WT muscle is comparable to that recently reported for murine m. soleus (30 pN*nm\(^{-1}\)*μm\(^{-1}\); ref. 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. Figure 5, C and D, suggests 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 Figure 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 Ca\textsuperscript{2+} 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 that 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 into the mechanisms underlying the previously reported fast relaxation kinetics of myofibrils from nebulin-deficient mice (36): in the absence of nebulin, the thin filaments were 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 would also increase the calcium sensitivity of force, as indeed observed in NEM6 fibers (Supplemental Figure 2, D and E) and indirectly in muscle of Kbtbd13\textsuperscript{R408C}\textsuperscript{−}KI mice (Supplemental Figure 5H). We speculate that the KBTBD13\textsuperscript{R408C}–induced increased flexural rigidity of thin filaments might play a role (Figure 6E; 30% increase in the persistence length of actin filaments exposed to recombinant KBTBD13\textsuperscript{R408C} protein). This speculation is based on previous reports indicating that, similarly to KBTBD13\textsuperscript{R408C}, the actin-binding protein phalloidin (a) increases the persistence length of actin filaments (37) with (b) 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 modeling suggested that KBTBD13 binds to actin (Figure 6A), which was confirmed by pull-down assays and cosedimentation assay with filamentous actin (Figure 6, B and C). APEX-based EM of GFP-tagged mutant and WT KBTBD13 in zebrafish showed localization consistent with KBTBD13 binding to actin in intact muscle fibers (Figure 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 tropomysin and tropinin to actin (both 1:7). Tropomysin, tropinin, and 7 actin monomers together form one regulatory unit, and a serial sequence of these regulatory units control thin-filament activation and deactivation. This raises the intriguing possibility of KBTBD13 acting in concert with actin, tropomysin, and tropinin to fine-tune the kinetics of muscle contractility. It should be noted that the APEX-based EM studies in zebrafish suggested that KBTBD13 also localizes to the cytoplasm and the thick filament (Figure 6D). Indeed, the low-angle x-ray diffraction studies in Kbtbd13–KO mice showed reduced thick-filament backbone periodicities (M6 and 2.8 nm reflections; Supplemental Figure 5, K and L), suggesting an altered structure. Thick-filament stiffness, however, was unaffected, as indicated by the unaltered Δ2.8 nm reflection during muscle activation (Supplemental Figure 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 “premyofibrils” of c2c12 cells, which suggested cytosolic localization, but no myofibrillar localization (20). Perhaps localization of KBTBD13 depends on the developmental stage of muscle and KBTBD13 doesn’t localize to the actin filament until after the premyofibril stage.

To date, 3 disease-causing mutations in KBTBD13 have been described, of which the p.R408C mutation is the most frequently described one. Here, we report 2 mutations in KBTBD13: p.I369M and p.E83Q. Notably, I369 is located near C367 (Figure 6A). This suggests that the mechanism underling the observed slowing of relaxation kinetics of human muscle fibers exposed to KBTBD13\textsuperscript{R408C} (Figure 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\textsuperscript{R408C} is a gain-of-function mutation. This proposition is based on the observation that (a) the Kbtbd13\textsuperscript{R408C}–KI mice do not phenocopy the mice that are deficient in KBTBD13 (e.g., unlike the Kbtbd13–KO mice, the Kbtbd13\textsuperscript{R408C} mice have slower kinetics of muscle relaxation; Figure 5H); and (b) KBTBD13\textsuperscript{wt} protein did not affect the flexural rigidity of thin filaments, whereas KBTBD13\textsuperscript{R408C} increased the rigidity (Figure 6E). Thus, KBTBD13\textsuperscript{wt} protein does...
not affect thin-filament structure and muscle-relaxation kinetics, whereas KBTBD13R408C 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 (Figure 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 KBTBD13R408C protein (Figure 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 4 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 (Figure 7). We propose that this pathomechanism is central to the NEM6 phenotype.

Methods

Please see the Supplemental Methods for a detailed description of TMS, muscle biopsies, permeabilized muscle-fiber mechanics, myosin heavy chain isoform composition determination, myofibril mechanics, recombinant KBTBD13 and KBTBD13R408C protein expression, actin and KBTBD13/KBTBD13R408C cosedimentation assay, pull-down assay with recombinant KBTBD13 proteins, mass spectrometry, KBTBD13 and KBTBD13R408C incubation assay in human muscle fibers, generation of the Kbtbd13-KO and Kbtbd12R408C K1 mouse models, intact muscle mechanics, homology modeling, KBTBD13 localization in zebrafish, antibodies used for Western blot, flexural rigidity of actin filaments, EM, immunohistochemistry, Ca$^{++}$ reuptake, low-angle x-ray diffraction, and stimulated emission-depletion microscopy.

Statistics. Data are presented as mean ± SEM or as median with interquartile range (normally distributed, not normally distributed, respectively). For statistical analyses, 2-tailed t tests or Mann-Whitney U tests were used. A P value of less than 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 for 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 contributions

JMDW, CACO, DER, BB, RAB, RJBR, and TI designed research studies. JMDW, JPM, MY, RVDP, SS, SC, MVDL, MW, SJBP, ESBVK, SL, MP, TES, AAR, VO, GR, TEH, XZ, CNJ, FL, B Kiss, NLV, RAB, MM, LN, RJJR, B Kösters, JD, KC, WM, and EM conducted experiments. JMDW, JPM, MY, RVDP, SS, SC, MVDL, MW, SJBP, ESBVK, SL, MP, TES, AAR, VO, GR, TEH, XZ, CNJ, FL, B Kiss, NLV, RAB, MM, LN, RJJR, B Kösters, JD, KC, WM, and EM acquired data. JMDW, JPM, CACO, NCV, BGMVE, HG, AHB, and NR wrote the manuscript. Both JMDW and JPM provided the majority of work in this study: JMDW took responsibility for the preclinical part and JPM for the clinical work. As during the study progress, the preclinical part increased significantly over the clinical work, JMDW is now listed as first author and JPM as second.

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