Tropomodulin1 directly controls thin filament length in both wild-type and tropomodulin4-deficient skeletal muscle

David S. Gokhin¹, Julien Ochala², Andrea A. Domenighetti³,⁴, Velia M. Fowler¹

¹ Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA
² Centre of Human and Aerospace Physiological Sciences, King’s College London, London SE1 1UL, UK
³ Department of Physical Medicine and Rehabilitation, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA
⁴ Rehabilitation Institute of Chicago, Chicago, IL 60611, USA

Author and address for correspondence:
Velia M. Fowler, Ph.D.  David S. Gokhin, Ph.D.
Department of Cell and Molecular Biology  Department of Cell and Molecular Biology
The Scripps Research Institute  The Scripps Research Institute
10550 N Torrey Pines Rd, MB114  10550 N Torrey Pines Rd, MB114
La Jolla, CA 92037  La Jolla, CA 92037
Phone: 858-784-8277  Phone: 858-784-8059
Fax: 858-784-8753  Fax: 858-784-9090
Email: velia@scripps.edu  Email: dgokhin@scripps.edu

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ABSTRACT

Sarcomeric tropomodulin (Tmod) isoforms, Tmod1 and Tmod4, cap thin filament pointed ends and functionally interact with leiomodin (Lmod) isoforms, Lmod2 and Lmod3, to control myofibril organization, thin filament lengths, and actomyosin crossbridge formation in skeletal muscle fibers. Here, we show that Tmod4 is more abundant than Tmod1 on both the transcript and protein levels in a variety of muscle types, but the relative abundances of sarcomeric Tmods are muscle-specific. We then generate Tmod4−/− mice, which exhibit normal thin filament lengths, myofibril organization, and skeletal muscle contractile function due to compensatory upregulation of Tmod1, together with an Lmod isoform switch wherein Lmod3 is downregulated and Lmod2 is upregulated. However, RNAi depletion of Tmod1 from either wild-type or Tmod4−/− muscle fibers leads to thin filament elongation by ~15%. Thus, Tmod1 per se, rather than total sarcomeric Tmod levels, controls thin filament lengths in mouse skeletal muscle, while Tmod4 appears to be dispensable for thin filament length regulation. These findings identify Tmod1 as the key direct regulator of thin filament length in skeletal muscle, in both adult muscle homeostasis and in developmentally compensated contexts.
INTRODUCTION

The semicrystalline arrays of interdigitating, uniform-length actin (thin) and myosin (thick) filaments found in the contracting sarcomeres of skeletal muscle fibers represent a striking example of long-range cytoskeletal organization and precise organelle size control. While thick filament lengths are essentially constant in all skeletal muscles and species examined (~1.65 μm), thin filament lengths vary substantially across muscles and vertebrate species (0.95-1.40 μm) (Castillo et al., 2009; Gokhin et al., 2012; Gokhin et al., 2010; Granzier et al., 1991; Ringkob et al., 2004). Thin filament lengths are remarkably plastic during normal postnatal skeletal muscle development and aging (Gokhin et al., 2014a) but can become misspecified in some congenital myopathies. For example, thin filaments are abnormally short due to aberrant F-actin assembly and/or destabilization in some nemaline myopathies (Ochala et al., 2012; Ottenheijm et al., 2010; Ottenheijm et al., 2009; Yuen et al., 2014), while thin filaments elongate due to pointed-end uncapping and actin subunit addition in mouse models of Duchenne muscular dystrophy (Gokhin et al., 2014b). Misspecification of thin filament lengths frequently occurs with alterations in thin filament activation and/or actomyosin crossbridge formation (Chandra et al., 2009; Ochala et al., 2012; Ottenheijm et al., 2013; Ottenheijm et al., 2010).

Critical players in the simultaneous regulation of thin filament lengths, thin filament activation, and actomyosin crossbridge formation are the tropomodulin (Tmod) family of tropomysin (TM)-binding and actin filament pointed-end capping proteins. Vertebrate skeletal muscles contain two sarcomeric Tmod isoforms: Tmod1, expressed in diverse, terminally differentiated cells, including striated muscle, but also erythrocytes, neurons, and ocular lens fiber cells; and Tmod4, which is exclusive to skeletal muscle fibers (Almenar-Queralt et al., 1999; Conley et al., 2001; Gokhin and Fowler, 2011b; Yamashiro et al., 2012). Two sarcomeric Tmod molecules cap each thin filament pointed end and bind to the N-termini of the terminal α/βTM molecules on the thin filaments (Almenar-Queralt et al., 1999; Fowler et al., 1993; Gokhin and Fowler, 2011b; Gokhin et al., 2012; Gokhin et al., 2010; Gokhin et al., 2014b). Tmod4 is ~10-fold more abundant than Tmod1 in a mixture of isolated tibialis anterior (TA) and extensor digitorum longus (EDL) myofibrils (Gokhin et al., 2014b), indicating that Tmod4 is likely the predominant pointed-end cap in skeletal muscle thin filaments, at least in fast muscle. In addition, an extrasarcomeric Tmod isoform, Tmod3, caps the pointed ends of cytoplasmic γ-actin filaments in a sarcoplasmic reticulum (SR)-
associated cytoskeletal network (Gokhin and Fowler, 2011a). Skeletal muscle also contains two leiomodin (Lmod) isoforms, Lmod2 and Lmod3, which are larger Tmod family members that nucleate F-actin assembly and can antagonize Tmods’ capping activity (Chereau et al., 2008; Conley et al., 2001; Tsukada et al., 2010; Yuen et al., 2014).

Tmod perturbation studies in cultured cardiomyocytes and Drosophila indirect flight muscle have demonstrated that sarcomeric Tmods control thin filament lengths by dynamically regulating actin subunit association/dissociation from pointed ends (Bliss et al., 2014; Gregorio et al., 1995; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001; Sussman et al., 1998) and by stabilizing pointed ends via their interactions with terminal α/βTMAs (Mudry et al., 2003). A role for sarcomeric Tmods in regulating thin filament lengths has also been implicated in mammalian skeletal muscle, where calpain-mediated proteolysis of sarcomeric Tmods in dystrophic muscle is associated with thin filament elongation (Gokhin et al., 2014b), although potential indirect effects due to non-Tmod-related mechanisms have not been ruled out. However, recent analyses of mouse knockouts have uncovered more complex functions of sarcomeric Tmods in mammalian skeletal muscle contractility, as well as unexpected compensatory mechanisms. In Tmod1−/− muscle, Tmod3 compensates for absence of Tmod1 by translocating from its SR compartment to the thin filament pointed ends to preserve normal skeletal muscle development, myofibril assembly, and thin filament lengths (Gokhin and Fowler, 2011a; Gokhin et al., 2010). The switch from pointed-end capping by Tmod1 and Tmod4 in wild-type muscle to pointed-end capping by Tmod3 and Tmod4 in Tmod1−/− muscle impairs initial α/βTM movement during thin filament activation, inhibiting formation of productive actomyosin crossbridges and depressing isometric force production (Ochala et al., 2014).

While analyses of dystrophic and Tmod1−/− skeletal muscles have provided substantial insights into how sarcomeric Tmods regulate thin filament lengths and muscle physiology, direct evidence for Tmod-mediated regulation of skeletal muscle thin filament lengths has not been found. Additionally, the ~10-fold greater abundance of Tmod4 than Tmod1 in TA and EDL myofibrils (Gokhin et al., 2014b) led us to hypothesize that Tmod4’s properties at pointed ends are distinct from those of Tmod1. Here, we report that Tmod4 is more abundant than Tmod1 on both the transcript and protein levels in both fast- and slow-switch muscles, but molar ratios of sarcomeric Tmods are muscle-specific. We then generated a novel Tmod4−/− mouse and unexpectedly discovered that Tmod4−/− muscles exhibit normal thin filament
lengths and contractile function due to compensatory upregulation of Tmod1, with accompanying downregulation of Lmod3 and upregulation of Lmod2. RNAi depletion of Tmod1 from either wild-type or Tmod4-/- muscles leads to actin subunit addition onto pointed ends and identical extents of thin filament elongation, indicating that Tmod1 and not Tmod4 is critical for thin filament length regulation in mouse skeletal muscles. These findings demonstrate that pointed-end capping by sarcomeric Tmods directly controls thin filament lengths in both adult muscle homeostasis and in developmentally compensated contexts.
RESULTS

**Gene expression and protein abundance of sarcomeric Tmods**

To determine whether the relative abundances of Tmod4:Tmod1 are fixed or variable across muscle types, we performed quantitative western blotting of diverse skeletal muscles (TA, soleus, and diaphragm) lysates and determined the molar ratio of sarcomeric Tmods in each muscle. Tmod4 was more abundant than Tmod1 in all muscles examined, but Tmod4 ranged from ~9-10-fold more abundant than Tmod1 in TA, ~4-fold more abundant in soleus, and ~3-fold more abundant in diaphragm (Table 1), indicating that sarcomeric Tmod isoform distributions are muscle type-dependent. In all muscles, ~2 sarcomeric Tmods were associated with each thin filament pointed end (Table 1), as observed previously (Gokhin et al., 2014b). Note that the higher Tmod1 levels observed in soleus and diaphragm muscles (Table 1) might be due to the heavier vascularization of these muscles and presence of Tmod1-containing erythrocytes (Moyer et al., 2010). However, this is unlikely, as we do not observe noteworthy differences in Tmod1 abundances in isolated myofibrils vs. whole-muscle lysates (compare Table 1 and (Gokhin et al., 2014b)). Alternatively, slow muscles such as the soleus and diaphragm might contain moreextrasarcomeric Tmod1 than fast muscles, possibly associated with the SR or T-tubules (Gokhin and Fowler, 2011a), leading to higher total Tmod1 levels.

Next, to determine whether greater abundance of Tmod4 as compared to Tmod1 in various skeletal muscles could be explained by differences in gene expression, we analyzed expression of *Tmod* genes in diverse striated muscles (TA, soleus, diaphragm, and cardiac left ventricle (LV)) using RNA-seq. *Tmod4* mRNA was more abundant than *Tmod1* mRNA in TA, soleus, and diaphragm (Fig. 1A), consistent with the higher levels of Tmod4 protein in these muscles (Table 1) and confirmed by qRT-PCR (Fig. 1B). In contrast, *Tmod1* mRNA was more abundant than *Tmod4* mRNA in LV (Fig. 1A), as expected (Gokhin and Fowler, 2011b). Therefore, muscle-specific molar ratios of Tmod4:Tmod1 proteins correlate with mRNA levels.

To complete our survey of Tmod family gene expression, we examined expression of nonsarcomeric *Tmod* genes, as well as *Lmod* genes. Expression of *Tmod3* mRNA was low but above-background in all striated muscles examined (Fig. 1A), consistent with Tmod3’s
SR association with γcyto-actin (Gokhin and Fowler, 2011a), an actin isoform that is ~1/4000th as abundant as the αsk-actin that comprises the thin filaments (Hanft et al., 2006). Lmod2 and Lmod3 mRNAs were detected in all striated muscles, in agreement with previous studies (Cenik et al., 2015; Chereau et al., 2008; Conley et al., 2001; Nworu et al., 2015; Tian et al., 2015; Yuen et al., 2014), but an unexpected muscle type dependence was observed, with Lmod2 mRNA predominating in slow-twitch soleus, diaphragm, and left ventricular muscles and Lmod3 mRNA more highly expressed than Lmod2 mRNA in fast-twitch TA muscle (Fig. 1C). Tmod1 and Tmod2 mRNAs were not detected in striated muscles (Fig. 1A,C), consistent with Tmod2 expression being restricted to neuronal tissues (Cox et al., 2003; Watakabe et al., 1996) and Lmod1 expression being restricted to smooth muscle (Conley et al., 2001; Nanda and Miano, 2012).

Deletion of Tmod4 induces compensatory upregulation of Tmod1 to maintain normal myofibril organization, thin filament lengths, and muscle function

Because Tmod4 and not Tmod1 is the predominant sarcomeric Tmod isoform in mouse skeletal muscles (Table 1), we explored the effects of Tmod4 deficiency in mice by generating Tmod4+/− mice, which lack Tmod4 protein in skeletal muscle (Fig. 2A-C). Surprisingly, Tmod4+/− mice were born in Mendelian ratios and were viable and fertile, with no overt myopathy, identical to Tmod4+/+ and Tmod4+/− mice. TA, EDL, and soleus muscles from Tmod4+/− mice exhibited no abnormalities in terms of centralized nuclei (Fig. 2D,E), fiber cross-sectional area (Fig. 2F), MHC distribution (Fig. 2G), myofibrillar ultrastructure (Fig. 2H), and physiological parameters Fig. 2I-L). Immunostaining for α-actinin and phalloidin staining for F-actin confirmed normal organization of striated arrays of Z-lines and thin filaments in Tmod4+/− muscles (Fig. 3A). Thus, Tmod4 appears dispensable for myofibril organization and skeletal muscle function in mice.

To test whether Tmod1 and/or Tmod3 could compensate for the absence of Tmod4 in Tmod4−/− mice, we immunostained longitudinal cryosections of Tmod4+/+ and Tmod4−/− muscles for Tmod1 and Tmod3. In both Tmod4+/+ and Tmod4−/− muscles, Tmod1 exhibited its familiar striated staining pattern of periodic doublets, corresponding to the pointed ends of phalloidin-stained thin filaments, but the fluorescence intensity of Tmod1 was qualitatively brighter in Tmod4−/− muscles, suggesting higher levels of Tmod1 (Fig. 3B, S1A). This was confirmed by western blotting, which showed ~6-8-fold increases in Tmod1 protein levels in Tmod4−/−
muscles (Fig. 3D,E), which was driven by ~5-6-fold increases in Tmod1 gene expression (Fig. 3F). Thus, the increased Tmod1 repopulated most of the pointed ends rendered vacant by the absence of Tmod4 (Table 1, Fig. 3H). Similar compensatory increases in Tmod1 protein levels were observed in leg and flank muscles from newborn (P0) Tmod4+/− mice (Fig. S2), indicating that compensatory elevation of Tmod1 levels occurs sometime during embryonic skeletal muscle development of Tmod4¢ mice and does not require postnatal muscle contractility and use. Unlike Tmod1, Tmod3 localized predominantly to Z-line-flanking puncta and to a lesser extent to M-line puncta in both Tmod4+/+ and Tmod4¢ muscles (Fig. 3C, S1B), indicative of Tmod3’s SR localization (Gokhin and Fowler, 2011a), but protein levels of Tmod3 were unchanged in Tmod4¢ muscles (Fig. 3D,E). Therefore, Tmod1 compensates for the absence of Tmod4 via an increase in protein levels. This compensation mechanism differs from Tmod1¢ muscle, wherein Tmod3 compensates for absence of Tmod1 via translocation from the SR and no change in protein levels (Gokhin and Fowler, 2011a; Gokhin et al., 2010). However, this compensation mechanism resembles Tmod2¢ brain, wherein Tmod1 protein levels are increased ~8-fold to compensate for the absence of Tmod2 in neurons (Cox et al., 2003).

Next, we asked whether the switch from Tmod1/Tmod4 pointed-end capping in Tmod4+/+ muscle to exclusively Tmod1 pointed-end capping in Tmod4¢ muscle might alter thin filament lengths. Hence, we performed distributed deconvolution (DDecon) analysis of line-scans from fluorescence images to measure thin filament lengths based on distances of sarcomeric Tmods from the Z-line as well as breadths of the F-actin striations (Gokhin and Fowler, 2013; Littlefield and Fowler, 2002). In both Tmod4+/+ and Tmod4¢ TA muscles, thin filament lengths were ~1.07 µm determined by Tmod distance and ~0.99 µm determined by F-actin breadth (Fig. 3G), in agreement with previous studies (Gokhin et al., 2010; Gokhin et al., 2014b). In both Tmod4+/+ and Tmod4¢ soleus muscles, thin filament lengths were ~1.18 µm determined by Tmod distance and ~1.10 µm determined by F-actin breadth (Fig. 3G). Therefore, compensatory upregulation of Tmod1 preserves normal thin filament lengths in Tmod4¢ muscle (Fig. 3H).

In skeletal muscle, thin filament lengths are stabilized by nebulin, a giant protein that coextends with thin filaments, protects thin filaments from depolymerization and shortening, and attenuates actin and Tmod dynamics at the pointed end (Bang et al., 2006; Pappas et al., 2011; Pappas et al., 2010). Nebulin extends from its C-terminus (neb-C) anchored in the Z-
line to its N-terminal M1M2M3 domain (neb-N) located a constant distance from the Z-line (~0.95 µm), slightly proximal to the pointed ends where sarcomeric Tmods are located (Gokhin and Fowler, 2013). Because nebulin and sarcomeric Tmods are thought to collaboratively regulate thin filament lengths, we asked whether deletion of Tmod4 might impact the assembly or localization of nebulin. To test this, we immunostained longitudinal cryosections of Tmod4+/+ and Tmod4−/− muscles for neb-N and neb-C. In both Tmod4+/+ and Tmod4−/− muscles, neb-N localized to striations slightly proximal to the thin filament pointed ends (Fig. 4A), as expected (Gokhin and Fowler, 2013), while neb-C precisely colocalized with α-actinin in the Z-line (Fig. 4B), also as expected (Pappas et al., 2008; Pappas et al., 2010). The neb-N striations in both TA and soleus muscles from Tmod4+/+ and Tmod4−/− mice were ~0.95 µm from the Z-line, as confirmed by DDecon analysis (Fig. 4D). Therefore, deletion of Tmod4 does not impact nebulin localization.

**Deletion of Tmod4 alters the expression of striated muscle Lmod isoforms**

Tmod4 has been proposed to functionally interact with the actin nucleator Lmod3 near the thin filament pointed ends, based on a dramatic decrease in Tmod4 protein levels in Lmod3-deficient nemaline myopathy patients (Yuen et al., 2014), and the ability of Tmod4 and Lmod3 to substitute for one another during skeletal myofibril assembly in *Xenopus* (Nworu et al., 2015). To evaluate this functional interaction, we examined whether deletion of Tmod4 might impact the localization or levels of Lmod3. In both Tmod4+/+ and Tmod4−/− muscles, Lmod3 localized to striations at or near the thin filament pointed ends (Fig. 4C), as expected (Nworu et al., 2015; Yuen et al., 2014). The Lmod3 striations were ~0.95 µm from the Z-line (Fig. 4D), coincident with neb-N and not the thin filament pointed end where Tmods are located (compare to Fig. 3G). We were also unable to detect Tmod-Lmod associations by coimmunoprecipitation (Fig. S3).

Surprisingly, western blotting of Tmod4−/− muscle lysates revealed a ~60% decrease in Lmod3 protein levels, with a corresponding increase in Lmod2 (Fig. 4E,F). These changes in Lmod isoform levels are due to corresponding changes in Lmod3 and Lmod2 mRNA levels (Fig. 4G). Therefore, the switch from Tmod1/Tmod4 pointed-end capping in Tmod4+/+ muscle to solely Tmod1 pointed-end capping in Tmod4−/− muscle shifts Lmod isoform abundance away from Lmod3 and toward Lmod2. However, since Tmods neither colocalize nor coimmunoprecipitate with Lmods, preferential Tmod4/Lmod3 and Tmod1/Lmod2
coexpression is unlikely to be regulated at the level of a protein complex; instead, Tmod4/Lmod3 vs. Tmod1/Lmod2 coexpression may be mediated by a gene-regulatory network, like other combinations of muscle cytoskeletal protein isoforms (Olson and Nordheim, 2010; Schiaffino and Reggiani, 1996).

Depletion of Tmod1 from wild-type or Tmod4-deficient muscles results in increased thin filament lengths

A direct role for sarcomeric Tmods in regulating thin filament length in mammalian skeletal muscle has remained elusive, due to developmental compensation by other Tmod isoforms in Tmod1/- and Tmod4/- muscles (Fig. 3 and (Gokhin and Fowler, 2011a; Gokhin et al., 2010)). To overcome this, we depleted Tmod1 from adult muscles using in vivo RNAi, in which we injected Tmod4+/+ and Tmod4/- TA muscles with either negative-control siRNA (siScr) or one of two Tmod1-targeting siRNAs (siTmod1a and siTmod1b). Muscle fibers from siScr-injected TA muscles consistently exhibited striated Tmod1 staining, whereas fibers from siTmod1a- and siTmod1b-injected muscles showed widespread loss of striated Tmod1 staining in many fibers (Fig. 5A,B; Fig. S4). Extent of knockdown was quantified as the percentage of fibers with striated Tmod1 staining, which was ~100% for siScr-injected TA muscles but reduced to ~25-50% for siTmod1a- and siTmod1b-injected muscles (Fig. 5C).

The effects of siTmod1a and siTmod1b injection were similar in Tmod4+/+ and Tmod4/- muscles (Fig. 5A-C), despite substantially higher Tmod1 mRNA levels and Tmod1 protein levels in Tmod4/- muscles prior to injection (Fig. 3). Tmod3 remained localized to Z-line-flanking and M-line puncta in siTmod1a- and siTmod1b-injected muscles (Fig. S5A,B), indicating that siRNA knockdown of Tmod1 does not induce compensatory translocation of Tmod3 from the SR to the thin filament pointed ends, as observed in Tmod1/- muscle (Gokhin and Fowler, 2011a; Gokhin et al., 2010).

Tmod1-negative fibers in siTmod1a- and siTmod1b-injected TA muscles exhibited a wider breadth of sarcomeric F-actin staining, indicative of increased thin filament lengths (Fig. 5A,B, yellow brackets). Indeed, thin filament lengths in Tmod1-positive fibers were ~0.98 µm, while thin filament lengths in Tmod1-negative knockdown fibers increased to ~1.13 µm (Fig. 5D). This ~15% increase is slightly greater than the ~10% increase observed in dystrophic TA muscles exhibiting m-calpain-mediated proteolysis of Tmod1 (Gokhin et al., 2014b), suggesting that residual unproteolyzed Tmod1 remaining in dystrophic TA muscle
might still confer partial length-regulating activity. Neb-N remained normally localized at ~0.95 μm from the Z-line in siTmod1a- and siTmod1b-injected muscles (Fig. S5C-E), indicating that overall thin filament elongation was driven specifically by elongation of the nebulin-free distal segment and not the nebulin-coated proximal segment (Gokhin and Fowler, 2013). Depletion of Tmod1 from Tmod4+/+ fibers did not affect variability of thin filament lengths (Fig. 5D and data not shown), even though the abundance of Tmod1 indicates it caps just a subset (~10%) of thin filaments (Table 1). Notably, Tmod4 remained normally localized at the pointed ends of the elongated thin filaments in siTmod1a- and siTmod1b-injected Tmod4+/+ muscles (Fig. 6A,C), indicating that Tmod4 acts as a dynamic cap, similar to Tmod1 (Gregorio et al., 1995; Littlefield et al., 2001). Moreover, Lmod3 remained ~0.95 μm from the Z-line in siTmod1a- and siTmod1b-injected Tmod4+/+ muscles (Fig. 6B,C), coincident with Neb-N (Fig. S5C-E), indicating that Lmod3 does not translocate to the pointed end to promote thin filament elongation in the absence of Tmod1.

Increased thin filament lengths in Tmod1-negative fibers were associated with increased sarcomere lengths at an ankle joint angle corresponding to maximal plantarflexion (Fig. 5E), suggesting that thin filament elongation drives sarcomere growth by inducing longitudinal separation of successive Z-lines. Coordination of thin filament length with sarcomere length has been observed during growth of Drosophila indirect flight muscle (Mardahl-Dumesnil and Fowler, 2001) but has unclear relevance to mammalian muscle (Littlefield and Fowler, 2008). It remains unknown whether thin filament elongation might drive sarcomere length changes during mammalian muscle development, and whether thin filament length changes might underlie sarcomere length changes under conditions of muscle adaptation and remodeling (Lieber, 2002).
DISCUSSION

Here, we show that Tmod4 is the predominant sarcomeric Tmod at both the transcript and protein levels in both fast and slow skeletal muscles, but Tmod4:Tmod1 ratios are muscle-specific. Muscle-specific sarcomeric Tmod isoform distributions represent a novel, myofibril-intrinsic signature of muscle types, similar to MHC, α/βTM, and troponin isoform distributions and Z-line widths (Pette and Staron, 1990). Although Tmod4 is the predominant Tmod in all muscles examined, deletion of Tmod4 does not produce an overt myopathy, since normal myofibril organization, thin filament lengths, and actomyosin contractile function are observed in Tmod4-/- skeletal muscle. This is likely due to compensatory upregulation of Tmod1 of sufficient magnitude to repopulate nearly all the thin filament pointed ends available upon Tmod4 deletion, which occurs during embryonic skeletal muscle development and does not require postnatal muscle use. Such a compensation mechanism appears to be a mammalian innovation, seeing as Tmod4 deficiency leads to severe myofibril disruption and nemaline myopathy-like phenotypes in zebrafish and Xenopus (Berger et al., 2014; Nworu et al., 2015). The dispensability of Tmod4 for mammalian skeletal muscle development and organization also agrees with the fact that no human TMOD4-based nemaline myopathies have been identified to date, despite that fact that numerous nemaline myopathy-causing mutations in other thin filament structural components and actin-associated proteins have been identified (Romero et al., 2013).

Tmod regulation of thin filament lengths

Our finding that RNAi depletion of Tmod1 leads to thin filament elongation in both wild-type and Tmod4-deficient TA muscle fibers provides the first direct demonstration that pointed-end capping by a sarcomeric Tmod isoform is inversely related to thin filament length in mammalian skeletal muscle fibers. While it has been known for some time that pointed-end capping by sarcomeric Tmods in inversely related to thin filament length in cardiac myocytes as well as Drosophila indirect flight muscle (Bliss et al., 2014; Gregorio et al., 1995; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001; Sussman et al., 1998), the only evidence for Tmod participation in thin filament length regulation in skeletal muscle has been indirect evidence obtained from dystrophic mouse muscles, in which m-calpain-induced proteolysis of Tmod1 is associated with thin filament elongation (Gokhin et al., 2014b).
However, the utility of dystrophic muscles for studying Tmod-mediated thin filament length regulation is constrained by the fact that residual unproteolyzed Tmod remains in muscle after Ca$^{2+}$ influx and m-calpain induction, and that the extent of m-calpain proteolysis is highly variable and depends on both the muscle type and the severity of the dystrophy (Gokhin et al., 2014b). Moreover, thin filament elongation in dystrophic muscle could be due to m-calpain proteolysis of non-Tmod components (Tidball and Spencer, 2000). The RNAi approach utilized here overcomes these limitations and illuminates a direct link between Tmod1-mediated capping and thin filament length regulation in both normal (wild-type) and developmentally compensated (Tmod4-deficient) contexts.

In wild-type muscles, only ~10% of filaments are capped by Tmod1 (with the remainder capped by Tmod4). Upon siRNA depletion of Tmod1 from wild-type muscles, Tmod4 now caps the pointed ends of all of the now-longer thin filaments. This is consistent with dynamic capping by Tmod4, as previously observed for Tmod1 in chick cardiac myocytes and quail skeletal myotubes (Gregorio et al., 1995; Littlefield et al., 2001; Pappas et al., 2010). In cardiac myocytes, thin filament lengths are thought to arise from competition between Tmod1 and actin subunits at pointed ends, and, thus, reduced total Tmod would be expected to allow more frequent actin subunit addition, leading to thin filament elongation (Gregorio et al., 1995; Littlefield et al., 2001). Thin filament elongation is unlikely to be due to lower affinity of Tmod4 for thin filament pointed ends as compared to Tmod1, since no differences have been observed between Tmod1 and Tmod4 with respect to their actin-capping and tropomyosin-actin-capping activities (Almenar-Queralt et al., 1999). Notably, length uniformity is maintained after thin filament elongation, indicative of a constitutive mechanism for equalization of thin filament lengths across the sarcomere; such a constitutive mechanism for organelle size control has previously been proposed for Chlamydomonas flagella (Ludington et al., 2012).

Despite compensatory upregulation of Tmod1 and normal thin filament lengths in Tmod4-deficient muscles, Tmod4-deficient muscles provide novel insights into mechanisms of Tmod-mediated regulation of actin pointed end dynamics and thin filament lengths. Unlike wild-type muscles, in which only ~10% of filaments are capped by Tmod1, Tmod4-deficient muscles have essentially 100% of their thin filaments capped by Tmod1. Surprisingly, regardless of these markedly different proportions of Tmod1 in wild-type and Tmod4-deficient muscles, RNAi depletion of the small amount of Tmod1 in wild-type muscle, where
90% of total Tmod remains at pointed ends, has an identical effect as depletion of Tmod1 from Tmod4-deficient muscle, where no Tmod remains at pointed ends (i.e., thin filament lengthening by ~15% in both cases). We suggest two possibilities to explain this apparent paradox: (1) Thin filament lengths might be inversely related to sarcomeric Tmod1+4 levels, but this relationship may not be stoichiometric. Rather, any depletion of sarcomeric Tmod1+4 levels leading to uncapped pointed ends might reduce total Tmod levels below a threshold required to inhibit actin subunit association and filament lengthening. (2) Alternatively, Tmod1-deficient sarcomeres from Tmod4-deficient muscles might actually have a greater propensity for thin filament elongation due to further reductions in total Tmod and increased actin subunit association kinetics as compared to Tmod1-deficient sarcomeres from wild-type muscles, but, in this latter case, excessive elongation of uncapped thin filaments might be blocked by a structural constraint or “ceiling” on thin filament elongation within the sarcomere. Myosin thick filaments, titin, and/or M-line components may contribute to such a ceiling, as suggested previously (Littlefield and Fowler, 1998).

**Relationship between Tmods and Lmods in striated muscles**

Deletion of Tmod4 with compensatory upregulation of Tmod1 during development leads to an unexpected Lmod isoform switch, with downregulation of Lmod3 and upregulation of Lmod2, suggesting the existence of a gene-regulatory network mediating preferential Tmod4-Lmod3 and Tmod1-Lmod2 coexpression. Preferential Tmod4-Lmod3 and Tmod1-Lmod2 coexpression is also supported by previous findings: (1) Lmod3 is the predominant Lmod isoform in skeletal muscle, in which skeletal myofibrils contain predominantly Tmod4 on thin filament pointed ends (this study and (Cenik et al., 2015)). (2) Lmod2 is the predominant Lmod isoform in cardiac muscle, in which cardiac myofibrils contain solely Tmod1 on thin filament pointed ends (Chereau et al., 2008; Conley et al., 2001; Gokhin and Fowler, 2011b; Skwarek-Maruszewska et al., 2010); (3) Lmod3-deficient nemaline myopathy patients exhibit dramatic loss of Tmod4 protein (Yuen et al., 2014). Although the genetic circuitry regulating preferential Tmod4-Lmod3 and Tmod1-Lmod2 coexpression remains unknown, a possible candidate is the MRTF/SRF pathway, which has been proposed to activate muscle-specific genes via a feedback loop initiated by Lmod3-mediated actin polymerization and depletion of soluble actin (Cenik et al., 2015). It is conceivable that coexpression of Tmod4-Lmod3 vs. Tmod1-Lmod2 leads to different soluble actin levels,
leading to different extents of MRTF activation, different extents of SRF/MEF2 activity, and expression of skeletal vs. cardiac combinations of cytoskeletal genes.

Our unexpected observation that Lmod3 localizes ~0.95 µm away from the Z-line in mouse skeletal muscle indicates that Lmod3 is coincident with neb-N at the junction between the nebulin-coated proximal segment and nebulin-free distal segment of the thin filament (Gokhin and Fowler, 2013). This Lmod3 localization is also similar to Lmod3 localization in some human muscle biopsies, although human Lmod3 localization is more heterogeneous and subject-specific, with variable Lmod3 staining observed along the length of the filament itself, and, in a subset of instances, coinciding with both neb-N and Tmod (Yuen et al., 2014). Our coimmunoprecipitation experiments argue against the existence of Tmod-Lmod complexes, although we cannot rule out the possibility that Tmod-Lmod interactions are weak, transient, and/or dependent on additional regulatory factors. Regardless, both observed Lmod3 localization patterns imply that Lmods exert at least some of their actin-regulatory effects on thin filaments by binding along filament sides. The identical positioning of Lmod3 and neb-N ~0.95 µm away from the Z-line also agrees with the fact that both Lmod3 and neb-N are substrates of the ubiquitination inhibitor Klhl40 (Garg et al., 2014). Lmod3/neb-N/Klhl40 might constitute a novel actin-remodeling complex along the side of the thin filament, at the junction between the nebulin-coated proximal segment and nebulin-free distal segment of the thin filament.
MATERIALS AND METHODS

Generation and genotyping of Tmod4−/− mice

Mouse embryonic stem cells with a lacZ/neo insertion in intron 2 of the Tmod4 gene were obtained from the Knockout Mouse Project repository (project ID 33375) at the University of California-Davis and stored in liquid N₂ until use. Blastocyst injection and implantation into pseudopregnant females were performed at the Murine Genetics Core at The Scripps Research Institute. Resulting male chimeras were bred to C57BL/6J females to obtain germline transmission. Intercrosses of Tmod4+/− mice were performed to generate Tmod4−/− mice and control Tmod4+/+ littermates. Presence of wild-type and mutant Tmod4 alleles was confirmed by automated qPCR genotyping of tail-snip biopsies (Transnetyx, Cordova, TN). Adult mice were sacrificed by isoflurane inhalation followed by cervical dislocation, while newborn (P0) mice were sacrificed isoflurane inhalation followed by decapitation. All experiments were performed according to NIH animal care guidelines, as approved and enforced by the Institutional Animal Care and Use Committee at The Scripps Research Institute.

Antibodies

Primary antibodies were as follows: rabbit polyclonal antiserum to chicken Tmod4 preadsorbed by passage through a Tmod1 Sepharose column (R3577, 1:25 for cryosections, 1:2500 for western blots; (Gokhin et al., 2010)), affinity-purified rabbit polyclonal anti-human Tmod1 (R1749, 3.1 µg/ml for cryosections; (Gokhin et al., 2010)), rabbit polyclonal antiserum to residues 340-359 of a human Tmod1 peptide (PA2211, 1:5000 for western blots; (Gokhin et al., 2012)), rabbit polyclonal antiserum to human Tmod3 preadsorbed by passage through a Tmod1 Sepharose column (R5168, 1:100 for cryosections, 1:1000 for western blots; (Gokhin et al., 2010)), affinity-purified rabbit polyclonal anti-nebulin M1M2M3 domain (NEB-1, 1:100 for cryosections; Myomedix, Mannheim, Germany), affinity-purified rabbit polyclonal anti-nebulin M160-164 domain (10 µg/ml for cryosections; a gift from Carol C. Gregorio, University of Arizona, Tucson, AZ), rabbit polyclonal anti-Lmod3 (14948-1-AP, 1:100 for cryosections, 1:1000 for western blots; Proteintech, Chicago, IL), rabbit polyclonal anti-Lmod2 (S-12, 1:200 for western blots; Santa Cruz Biotechnology, Dallas, TX), mouse monoclonal anti-Tmod1 (mAb9, 1:100 for cryosections; (Gregorio et al., 2010)).
mouse monoclonal anti-α-actinin (EA53, 1:100 for cryosections; Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-actin (C4, 1:10000 for western blots; EMD Millipore, Billerica, MA), and mouse monoclonal anti-GAPDH (1D4, 1:5000 for western blots; Novus Biologicals, Littleton, CO). Secondary antibodies were as follows: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200 for cryosections; Life Technologies, Carlsbad, CA), Alexa Fluor 647-conjugated goat anti-mouse IgG (1:200 for cryosections; Life Technologies), 800CW-conjugated goat anti-rabbit IgG (1:20,000 for western blots; LI-COR Biosciences, Lincoln, NE) and 680LT-conjugated goat anti-mouse IgG (1:20,000 for western blots; LI-COR Biosciences).

**Immunostaining and confocal microscopy**

TA and soleus muscles were relaxed, fixed, cryosectioned, and immunostained as described previously (Gokhin et al., 2014b). Alexa 488- or Alexa 647-conjugated secondary antibodies were supplemented with rhodamine-phalloidin (1:100; Life Technologies) to stain F-actin. Images of single optical sections were collected at RT on either a Bio-Rad Radiance 2100 laser-scanning confocal microscope mounted on a Nikon TE2000-U microscope, or on a Zeiss LSM780 laser-scanning confocal microscope mounted on a Zeiss Axio Observer.Z1 microscope. Images were acquired using 100× (1.4 NA) Plan-Apochromat oil-objective lenses with digital zoom of up to 3×. Images were processed with Volocity 6.3 (PerkinElmer, Waltham, MA), and image figures were constructed in Adobe Illustrator CS5.1.

**Thin filament length measurements**

DDecon was used to measure distances of sarcomeric Tmods, neb-N, and Lmod3 from the Z-line as well as the breadth of the F-actin (phalloidin) signal across the Z-lines of adjacent half-sarcomeres (I-Z-I arrays) using line-scans of fluorescence images, as described previously (Gokhin et al., 2012; Littlefield and Fowler, 2002). Tmod distances were determined from Tmod1 and Tmod4 localization in \( Tmod4^{+/+} \) muscles or just Tmod1 localization in \( Tmod4^{-/-} \) muscles. DDecon was performed using an open-source ImageJ plugin (available at http://www.scripps.edu/fowler/). Distances were calculated by converting pixel sizes into μm.
Histology

Transverse sections of TA, EDL, and soleus muscles were subjected to H&E and Gömöri trichrome staining. Images were collected on a Zeiss Axioskop bright-field microscope using a 10× (0.45 NA) air-objective lens (zoom = 2) and a Zeiss Axiocam CCD camera at RT. Centrally nucleated fibers were manually counted in H&E-stained images. Fiber cross-sectional area was determined by manual segmentation of fibers and converting enclosed pixel areas into μm².

Coimmunoprecipitation

Coimmunoprecipitation was performed as described previously (Gokhin and Fowler, 2011a). Briefly, mouse TA and EDL muscles homogenized in RIPA buffer supplemented with protease inhibitor cocktail (1:1000) and centrifuged at 10,000g. The resulting supernatant was divided into 1-ml aliquots, to which one of the following was added: 2 μg rabbit polyclonal anti-Tmod4 (R3577), 2 μg rabbit polyclonal anti–human Tmod1 (R1749), or 2 μg of preimmune control IgG. Antibody-bound complexes were absorbed to 100 μl μMACS Protein A-conjugated super-paramagnetic MicroBeads (Miltenyi Biotec, San Diego, CA) and incubated for 1 h on ice, followed by passage through a μColumn (Miltenyi Biotec) using a μMACS magnetic separator (Miltenyi Biotec). Beads were washed with RIPA buffer and eluted with 100 μl of 1× SDS sample buffer. The input extract (diluted in 2× SDS sample buffer) and eluted immunoprecipitates were then subjected to SDS-PAGE and western blotting, as described below.

Gel sample preparation and western blotting

For western blots of whole-muscle homogenates, TA, EDL, soleus, diaphragm, and neonatal leg and flank muscles were dissected in ice-cold PBS and homogenized with a Tekmar Tissumizer in 10 volumes of PBS supplemented with protease inhibitor cocktail (1:1000). Protein standards for quantitative western blotting were prepared as described previously (Gokhin et al., 2014b). First, protein standards for SDS-PAGE and Coomassie blue staining to determine actin concentrations in isolated myofibrils were prepared from rabbit skeletal muscle actin (Cytoskeleton, Denver, CO). Second, protein standards for quantitative western blots were prepared by spiking increasing volumes of Tmod1−/− (Gokhin et al., 2010) or
Tmod4−/− TA muscle homogenates with increasing amounts of recombinant purified human Tmod1 or mouse Tmod4 proteins (Yamashiro et al., 2010), respectively. This approach equalized the effects of endogenous non-Tmod proteins on the differential western transfer efficiencies of endogenous vs. recombinant purified Tmods, as described previously (Gokhin et al., 2014b).

All protein preparations were solubilized in equal volumes of 2× SDS sample buffer and boiled for 5 min. SDS-PAGE, transfer to nitrocellulose, blocking, and antibody incubations were performed as described previously (Gokhin et al., 2014b). Bands were visualized using a LI-COR Odyssey® infrared imaging system, and background-corrected band intensities were densitometrically quantified in ImageJ. Where appropriate, band intensities were normalized to GAPDH to control for loading.

**MHC isoform composition**

Gel samples of whole-muscle homogenates were prepared as described under “Western blotting” above. Proteins were then separated via SDS-PAGE on 8% Tris-glycine mini-gels, as described previously (Talmadge and Roy, 1993). SDS-PAGE was performed for 16 h at 140 V. The upper tank buffer was supplemented with 1 mM DTT, and the lower tank buffer was 1/6th the concentration of the upper tank buffer (Kohn and Myburgh, 2006). Gels were silver-stained, and background-corrected intensities of type-1, -2A, -2X, and -2B MHC bands were densitometrically quantified in ImageJ.

**Skinned muscle fiber mechanics**

Physiological measurement of specific force production, rate constant of force redevelopment after unloaded sarcomere shortening (ktr), thin filament Ca^{2+} sensitivity (pCa_{50}), and cooperativity of thin filament activation (Hill coefficient (nH)) using skinned, Ca^{2+}-activated TA muscle fibers was performed as described previously (Ochala et al., 2014).

**RNA sequencing**

RNA sequencing was performed as described previously (Christodoulou et al., 2011). Briefly, an RNA sequencing library was constructed via random hexamer priming of mRNA
pooled from three biological replicates of wild-type mouse TA, soleus, diaphragm, and left ventricular muscles (Domenighetti et al., 2014). The resulting cDNA library was uniformly amplified to subsaturating levels. Alignment of reads was performed using TopHat 1.0.14 (Trapnell et al., 2009). Read-depth profiles were constructed using TopHat's “wiggles” tool, and values were normalized to total aligned reads and uploaded onto the University of California-Santa Cruz Genome Browser (http://genome.ucsc.edu).

qRT-PCR

Total RNA was extracted from skeletal muscle tissues with TRIzol reagent (Life Technologies) and reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase with random hexamer primers (Life Technologies), according to the manufacturer’s instructions. cDNA was analyzed using a Bio-Rad iCycler iQ™ apparatus, with each reaction containing 60 ng cDNA as template, LightCycler® 480 SYBR Green I Master (Roche, Indianapolis, IN), and the following gene-specific primers (Integrated DNA Technologies, San Diego, CA):

- **Tmod4** forward, 5’-GATGCAGTAGAGATGGAGATG-3’;
- **Tmod4** reverse, 5’-TCTCTTTTTGCTGACGACG-3’;
- **Tmod1** forward, 5’-CAACGCCATGATGAGCAAC-3’;
- **Tmod1** reverse, 5’-CATCGGTAGAACACGTCCAG-3’;
- **Lmod3** forward, 5’-CCGCTGTTGGAGAATCACTCCC-3’
- **Lmod3** reverse, 5’-ACTCCAGCTCCTTTGCGACGTTG-3’
- **Lmod2** forward, 5’-TTGGAGAAGGAACGGCTGGG-3’
- **Lmod2** reverse, 5’-CCTCAGAGACTTCGCTGTTGCTCTC-3’
- **Gapdh** forward, 5’-ACCACAGTCCATGCCATCAC-3’;
- **Gapdh** reverse, 5’-TCCACCACCCTGGTTCGTA-3’.

*Tmod4* and *Tmod1* primers were as in (Gokhin et al., 2014b), *Lmod3* and *Lmod2* primers were as in (Cenik et al., 2015), and *Gapdh* primers were ReadyMade™ primers from Integrated DNA Technologies. Relative gene expression levels were calculated as $2^{-\Delta\Delta Ct}$ with normalization to *Gapdh*, as described previously (Livak and Schmittgen, 2001).
**In vivo RNAi**

Mice were anesthetized with isoflurane (4% for induction, 2% for maintenance) with 1 L/min O₂, until cessation of the toe-pin withdrawal reflex was attained. One nmol siRNA and Lipofectamine® RNAiMAX transfection reagent (1:100, Life Technologies) were diluted to a final volume of 10 µl, drawn into a 25-gauge syringe needle, and slowly injected into the midbelly of the TA muscle. Two different Silencer® Select siRNA constructs (Life Technologies) were used to knockdown Tmod1:

- siTmod1a forward, 5'-CUAGAAGAGGUUAAUCUUATT-3';
- siTmod1a reverse, 5'-UAAGAUUAACCUCUCUAGTT-3';
- siTmod1b forward, 5'-GCUGAAUGCUGAAAGUGATT-3';
- siTmod1b reverse, 5'-UCACUUUCAGCAUUUCAGCAA-3'.

As a negative control, a Stealth™ RNAi negative control duplex (Life Technologies) was used and is referred to as siScr. After injection, the ankle joint was passively dorsiflexed and plantarflexed to facilitate distribution of injectate throughout the TA, and mice were removed from anesthesia and allowed to recover on a heated pad. After 1 wk, TA muscles were excised and processed for immunostaining and confocal microscopy, as described above.

**Statistics**

Data are presented as either mean±s.d. or mean±s.e.m., where appropriate. Differences between groups were detected using Student’s t-test or one-way ANOVA with *post hoc* Fisher’s LSD tests, where appropriate. Statistical significance was defined as *p*<0.05. Analysis was performed in Microsoft Excel.
ACKNOWLEDGEMENTS

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REFERENCES


**Figure 1.** Gene expression of Tmod family members in striated muscles. (A) Expression of Tmod1-4 mRNAs in TA, soleus, diaphragm, and LV muscle determined by RNA-seq. (B) Expression of Tmod1 and Tmod4 mRNAs in TA and soleus muscles determined by qRT-PCR. Error bars reflect mean±s.e.m. of n=3 replicates. *, p<0.05. (C) Expression of Lmod1-3 mRNAs in TA, soleus, diaphragm, and LV muscle determined by RNA-seq. For RNA-seq analyses, 3 individual muscles of each type were pooled, and RNA-seq analysis was performed on the pooled sample; hence, no error bars are shown.
Figure 2. Deletion of Tmod4 does not produce an overt myopathic phenotype. (A) Tmod4 gene targeting strategy generated by KOMP, depicting LacZ/neo insertion into intron 2 of Tmod4. (B) Automated qPCR analysis of genomic DNA from mouse tail-snip biopsies derived from Tmod4+/− intercrosses. *, p<0.001 vs. other groups. (C) Western blots of homogenates of TA muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice probed using an anti-Tmod4 antibody. GAPDH was used as a loading control. (D) H&E-stained cross-sections of soleus muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice. Bars, 300 µm. (E-F) Histological evaluation of (E) centralized nuclei and (F) fiber cross-sectional area in TA, EDL, and soleus muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice. Error bars reflect mean±s.e.m. of n=4 muscles/genotype. (G) Evaluation of MHC isoform distributions in TA, EDL, and soleus muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice using 8% SDS-PAGE followed by silver staining and densitometric quantification. Error bars reflect mean±s.e.m. of n=4 muscles/genotype. (H) Representative TEM micrographs of longitudinal sections of TA and EDL muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice. Bars, 1 µm. (I-L)
Physiological evaluation of skinned, Ca\textsuperscript{2+}-activated muscle fibers from TA muscles from 2-mo-old $Tmod4^{+/+}$ and $Tmod4^{-/-}$ mice. Shown are (I) specific force production, (J) crossbridge cycling kinetics as determined by the rate constant of force redevelopment after unloaded sarcomere shortening ($k_tr$), (K) thin filament Ca\textsuperscript{2+} sensitivity ($pCa_{50}$), and (L) cooperativity of thin filament activation (Hill coefficient ($n_H$)). Error bars reflect mean±s.e.m. of $n$=8 fibers/genotype.
Figure 3. Deletion of Tmod4 induces compensatory upregulation of Tmod1 to preserve myofibril organization and thin filament lengths. (A-C) Longitudinal cryosections of TA muscles from 2-mo-old $Tmod4^{+/+}$ and $Tmod4^{-/-}$ mice were immunostained for either (A) Tmod4, (B) Tmod1, or (C) Tmod3, immunostained for α-actinin, and phalloidin-stained for F-actin. P, thin filament pointed ends; Z, Z-line; M, M-line. Bars, 1 µm. (D) Western blots of homogenates of TA, EDL, and soleus muscles from 2-mo-old $Tmod4^{+/+}$ and $Tmod4^{-/-}$ mice were probed using antibodies against Tmod4, Tmod1, Tmod3, and actin. GAPDH was used as a loading control. (E) Quantification of western blots. Error bars reflect mean±s.e.m. of $n=4$ lanes/genotype within a single blot. (F) Expression of $Tmod1$ mRNA in TA and soleus muscles from $Tmod4^{+/+}$ and $Tmod4^{-/-}$ mice determined by qRT-PCR. Error bars reflect
mean±s.e.m. of n=3 replicates. *, p<0.05. (G) Thin filament lengths in TA and soleus muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice determined using DDecon analysis of fluorescence images. Parameters correspond to the breadth of phalloidin staining and the distances of Tmod and nebulin M1M2M3 from the Z-line. In Tmod4+/+ muscle, Tmod lengths were determined from both Tmod1 and Tmod4 immunostaining. In Tmod4−/− muscle, Tmod lengths were determined from only Tmod1 immunostaining. Error bars reflect mean±s.d. of n=50 myofibrils/genotype randomly selected from n=4 muscles/genotype. (H) Possible model of sarcomeric Tmod isoform association with thin filament pointed ends in sarcomeres from Tmod4+/+ and Tmod4−/− muscles.
Deletion of Tmod4 induces downregulation of Lmod3 with concomitant upregulation of Lmod2, but does not impact the localization of nebulin. (A-C) Longitudinal cryosections of TA muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice were immunostained for either (A) neb-N, (B) neb-C, or (C) Lmod3, immunostained for α-actinin, and phalloidin-stained for F-actin. P, thin filament pointed ends; Z, Z-line. Bars, 1 μm. (D) Distances of neb-N and Lmod3 from the Z-line in TA and soleus muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice determined using DDecon analysis of fluorescence images. Error bars reflect mean±s.d. of n=50 myofibrils/genotype randomly selected from n=4 muscles/genotype. (E) Western blots of homogenates of TA muscles from 2-mo-old Tmod4+/+ and Tmod4−/− mice were probed using antibodies against Lmod3 and Lmod2. GAPDH was used as a loading control. (F) Quantification of western blots. Error bars reflect
mean±s.e.m. of \( n=4 \) lanes/genotype within a single blot. (G) Expression of \( Lmod3 \) and \( Lmod2 \) mRNA in TA muscles from \( Tmod4^{+/+} \) and \( Tmod4^{-/-} \) mice determined by qRT-PCR. Error bars reflect mean±s.e.m. of \( n=3 \) replicates. *, \( p<0.05 \).
Figure 5. RNAi knockdown of Tmod1 from \( Tmod4^{+/+} \) or \( Tmod4^{-/-} \) muscles leads to increased thin filament lengths. (A-B) TA muscles from 2-mo-old (A) \( Tmod4^{+/+} \) and (B) \( Tmod4^{-/-} \) mice were injected with siScr, siTmod1a, or siTmod1b and excised 1 week post-
injection. Shown are longitudinal cryosections immunostained for Tmod1 and α-actinin, and phalloidin-stained for F-actin. For siTmod1a, images of a muscle fiber away from the siRNA injection site ("Tmod1-positive cell") are also shown, serving as an internal control. Yellow brackets signify thin filament arrays (I-Z-I regions) that widen after Tmod1 knockdown. P, thin filament pointed ends; Z, Z-line. Bars, 1 µm. (C) Extent of Tmod1 knockdown as measured by the percentage of fibers with striated Tmod1 immunostaining in TA muscles injected with siScr, siTmod1a, or siTmod1b. Error bars reflect mean±s.e.m. of n=3-4 muscles. (D-E) Measurement of (D) thin filament lengths and (E) sarcomere lengths in TA muscles at maximal plantarflexion in 2-mo-old Tmod4+/− and Tmod4−/− mice injected with siScr, siTmod1a, or siTmod1b, determined using DDecon analysis of fluorescence images. Error bars reflect mean±s.d. of n=50 myofibrils/genotype randomly selected from n=3-4 muscles/genotype. *, p<0.01 vs. siScr.
Figure 6. RNAi knockdown of Tmod1 from wild-type muscle does not alter the localization of Tmod4 or Lmod3. (A-B) TA muscles from 2-mo-old Tmod4+/+ mice were injected with siScr, siTmod1a, or siTmod1b and excised 1 week post-injection. Shown are longitudinal cryosections immunostained for Tmod1 and either (A) Tmod4 or (B) Lmod3, and phalloidin-stained for F-actin. Yellow brackets signify thin filament arrays (I-Z-I regions) that widen after Tmod1 knockdown. P, thin filament pointed ends; Z, Z-line. Bars, 1 µm. (B) Distances of Tmod4 and Lmod3 from the Z-line in TA muscles from 2-mo-old Tmod4+/+ mice injected with siScr, siTmod1a, or siTmod1b, determined using DDecon analysis of fluorescence images. Error bars reflect mean±s.d. of n=50 myofibrils/genotype randomly selected from n=3-4 muscles/genotype. *, p<0.01 vs. siScr.
### TABLE 1

**Stoichiometry of sarcomeric Tmods associated with thin filaments in diverse mouse muscles**

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<th></th>
<th>ng Tmod1* / μl gel sample</th>
<th>ng Tmod4* / μl gel sample</th>
<th>ng actin** / μl gel sample</th>
<th>mol Tmod4 / mol Tmod1</th>
<th>mol actin / mol sarc. Tmods</th>
<th>sarc. Tmods / thin filament***</th>
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<td></td>
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<td>980±48</td>
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* Amounts of sarcomeric Tmods were determined by quantitative western blotting and densitometry of skeletal muscle myofibrils (1-8 μl gel sample) electrophoresed alongside purified recombinant protein standards (0.25-4 ng) on the same gel. Tmod1 or Tmod4 protein standards were mixed with Tmod1+/− or Tmod4+/− TA muscle lysates, respectively, to equalize the effects of endogenous non-Tmod proteins on the western transfer efficiencies of endogenous vs. recombinant purified Tmods. Standard deviations reflect 3-4 different myofibril volumes from different lanes on the same blot.

** Amounts of actin were determined by densitometry of Coomassie blue-stained gels containing skeletal muscle myofibrils (1-8 μl gel sample) electrophoresed alongside rabbit
skeletal muscle actin standards (0.25-4 μg) on the same gel. Standard deviations reflect 3-4 different myofibril volumes from different lanes on the same gel.

*** Numbers of Tmods/thin filament were calculated based on 13 actin subunits per 37 nm of thin filament (Fowler et al., 1993).