Calpain-mediated proteolysis of tropomodulin isoforms leads to thin filament elongation in dystrophic skeletal muscle

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a hereditary muscle-wasting disorder afflicting ~1 in 3500 males. DMD is caused by absence of the costameric protein dystrophin from the sarcolemma of skeletal muscle fibers, leading to sarcolemmal mechanical instability, membrane rupture during muscle contraction, pathological influx of extracellular Ca\(^{2+}\) into the muscle fiber interior, resultant hyperactivity of Ca\(^{2+}\)-dependent proteases, and widespread misregulated proteolysis of muscle cytoskeletal proteins (Blake et al., 2002; Rahimov and Kunkel, 2013). Key players in the progression of dystrophic muscle fibers, leading to sarcolemmal mechanical instability, membrane rupture during muscle contraction, pathological influx of extracellular Ca\(^{2+}\) into the muscle fiber interior, resultant hyperactivity of Ca\(^{2+}\)-dependent proteases, and widespread misregulated proteolysis of muscle cytoskeletal proteins (Blake et al., 2002; Rahimov and Kunkel, 2013). Key players in the progression of dystrophic skeletal muscle. These results implicate Tmod proteolysis and resultant thin filament length misspecification as novel mechanisms that may contribute to DMD pathology, affecting muscles in a use- and disease severity-dependent manner.

ABSTRACT

Duchenne muscular dystrophy (DMD) induces sarcolemmal mechanical instability and rupture, hyperactivity of intracellular calpains, and proteolytic breakdown of muscle structural proteins. Here we identify the two sarcomeric tropomodulin (Tmod) isoforms, Tmod1 and Tmod4, as novel proteolytic targets of m-calpain, with Tmod1 exhibiting ~10-fold greater sensitivity to calpain-mediated cleavage than Tmod4 in situ. In mdx mice, increased m-calpain levels in dystrophic soleus muscle are associated with loss of Tmod1 from the thin filament pointed ends, resulting in ~11% increase in thin filament lengths. In mdx/mTR mice, a more severe model of DMD, Tmod1 disappears from the thin filament pointed ends in both tibialis anterior (TA) and soleus muscles, whereas Tmod4 additionally disappears from soleus muscle, resulting in thin filament length increases of ~10 and ~12% in TA and soleus muscles, respectively. In both mdx and mdx/mTR mice, both TA and soleus muscles exhibit normal localization of α-actinin, the nebulin M1M2M3 domain, Tmod3, and cytoplasmic γ-actin, indicating that m-calpain does not cause wholesale proteolysis of other sarcomeric and actin cytoskeletal proteins in dystrophic skeletal muscle. These results implicate Tmod proteolysis and resultant thin filament length misspecification as novel mechanisms that may contribute to DMD pathology, affecting muscles in a use- and disease severity-dependent manner.

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Abbreviations used: DDecon, distributed deconvolution; DMD, Duchenne muscular dystrophy; EDL, extensor digitorum longus; γ-actin, cytoplasmic γ-actin; SR, sarcoplasmic reticulum; TA, tibialis anterior; Tmod, tropomodulin; WT, wild type.

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Given the complex regulatory mechanisms that govern calpain activity in vivo, direct evidence implicating specific intrasarcromeric calpain substrates in the pathogenesis and progression of DMD-induced muscle wasting is elusive. One potential intrasarcromeric target of calpain-mediated proteolysis in dystrophic muscle is the tropomodulin (Tmod) family of actin filament pointed-end–capping proteins. Tmods are dynamic caps that regulate actin subunit association and dissociation from pointed ends in a tropomyosin-dependent manner (Weber et al., 1994, 1999), thereby controlling actin filament lengths and stability in a diverse assortment of cell types (Yamashiro et al., 2012). In the case of skeletal muscle sarcomeres, two sarcomeric Tmod isoforms (Tmod1 and Tmod4) localize to either side of the M-line, along the periphery of the H-zone, where they cap the pointed (free) ends of the thin filaments (Fowler et al., 1993; Almenar-Queralt et al., 1999; Gokhin et al., 2010, 2012; Gokhin and Fowler, 2011b). Sarcomeric Tmods maintain thin filament stability and correctly specified thin filament lengths in Drosophila and vertebrate cardiac muscles via their interactions with terminal tropomysins and their ability to regulate actin subunit exchange at pointed ends (Gregorio et al., 1995; Sussman et al., 1998; Littlefield et al., 2001; Littlefield and Fowler, 2008; Mardahl-Dumesnil and Fowler, 2001; Mudry et al., 2003). In skeletal muscle, sarcomeric Tmods are proposed to fine-tune the lengths of the nebulin-free distal segments of the thin filaments, independent of the lengths of the nebulin-coated proximal segments of the thin filaments (Gokhin and Fowler, 2013). Given Tmods’ sterically accessible location within the sarcomere, as well as the fact that calpains can proteolyze a host of other actin-binding proteins in both muscle and nonmuscle cells (Carafoli and Molinari, 1998; Goll et al., 2003), we hypothesized that sarcomeric Tmods may be calpain substrates in DMD, with proteolysis of Tmods leading to misspecified thin filament lengths in dystrophic skeletal muscle. Such a mechanism would represent a novel and physiologically relevant defect in DMD because myofilament lengths determine the sarcomere length ranges at which muscles can establish actomyosin cross-bridges and produce contractile force (Gokhin and Fowler, 2013).

To directly address the question of whether calpain-mediated proteolysis of Tmods contributes to altered thin filament lengths and disease pathogenesis in DMD, we studied the responses of Tmods to calpain-mediated proteolysis in vitro and in dystrophic muscles in vivo. In this study, we show that both Tmod1 and Tmod4 are equally sensitive to calpain-mediated proteolysis in vitro, but Tmod1 is about an order of magnitude more sensitive than Tmod4 when associated with myofibrils in situ. In mdx mice, an increase in m-calpain levels in dystrophic muscle tropomysins (Gokhin et al., 2013), thereby controlling actin subunit exchange at pointed ends (Gregorio et al., 1995; Sussman et al., 1998; Littlefield et al., 2001; Littlefield and Fowler, 2008; Mardahl-Dumesnil and Fowler, 2001; Mudry et al., 2003). In skeletal muscle, sarcomeric Tmods are proposed to fine-tune the lengths of the nebulin-free distal segments of the thin filaments, independent of the lengths of the nebulin-coated proximal segments of the thin filaments (Gokhin and Fowler, 2013). Given Tmods’ sterically accessible location within the sarcomere, as well as the fact that calpains can proteolyze a host of other actin-binding proteins in both muscle and nonmuscle cells (Carafoli and Molinari, 1998; Goll et al., 2003), we hypothesized that sarcomeric Tmods may be calpain substrates in DMD, with proteolysis of Tmods leading to misspecified thin filament lengths in dystrophic skeletal muscle. Such a mechanism would represent a novel and physiologically relevant defect in DMD because myofilament lengths determine the sarcomere length ranges at which muscles can establish actomyosin cross-bridges and produce contractile force (Gokhin and Fowler, 2013). Given Tmods’ sterically accessible location within the sarcomere, as well as the fact that calpains can proteolyze a host of other actin-binding proteins in both muscle and nonmuscle cells (Carafoli and Molinari, 1998; Goll et al., 2003), we hypothesized that sarcomeric Tmods may be calpain substrates in DMD, with proteolysis of Tmods leading to misspecified thin filament lengths in dystrophic skeletal muscle. Such a mechanism would represent a novel and physiologically relevant defect in DMD because myofilament lengths determine the sarcomere length ranges at which muscles can establish actomyosin cross-bridges and produce contractile force (Gokhin and Fowler, 2013).

RESULTS

Sarcomeric Tmods are proteolytic substrates of m-calpain

Actin cytoskeletal proteins are frequently calpain cleavage substrates under a wide variety of pathological conditions (Carafoli and Molinari, 1998; Goll et al., 2003), but it is unknown whether sarcomeric proteins, such as Tmod1 and Tmod4, are also calpain cleavage substrates. To directly test whether m-calpain can proteolyze Tmod1 and/or Tmod4 in situ, we prepared isolated skeletal myofibrils from WT TA and extensor digitorum longus (EDL) muscles and treated these myofibrils with 40 μg/ml m-calpain activated by millimolar Ca2+. Immunostaining of m-calpain–treated myofibrils revealed disappearance of both Tmod1 and Tmod4 from the pointed ends of phalloidin-stained thin filaments in virtually all myofibrils examined, whereas the stained staining patterns of F-actin and α-actinin were unaffected (Figure 1, A and B). Western blotting confirmed depletion of Tmod1 and Tmod4 from the m-calpain–treated myofibrils, with no change in total actin levels, and Coomassie blue staining revealed no gross changes in myofibrillar protein composition resulting from m-calpain treatment (Figure 1C). Note that Tmod depletion determined by immunofluorescence appeared more striking than Tmod depletion determined by Western blotting, which may be due to either the narrower detection range of immunofluorescence or the inability of immunofluorescence to detect any Tmod protein that may have been extracted or solubilized from the washed myofibrils. We also examined myofibrils treated with either Ca2+ or m-calpain alone, as well as m-calpain in the presence of Ca2+ and a specific inhibitor (calpeptin). As expected, these control treatments had no effect on the localizations or protein levels of Tmod1 and Tmod4 (Figure 1, A–C).

Next we tested whether Tmod1 and Tmod4 have differential sensitivities to m-calpain–induced proteolysis in situ by treating myofibrils with increasing concentrations of m-calpain and performing Western blots for Tmod1 and Tmod4. Quantitation of these Western blots showed that myofibrillar Tmod1 had ∼10-fold greater sensitivity to m-calpain–induced proteolysis as compared with myofibrillar Tmod4 (Figure 1, D and E). This result contrasts with the similar sensitivities of purified recombinant Tmod1 and Tmod4 to m-calpain–induced proteolysis in vitro (Figure 2).

The differential susceptibilities of Tmod1 and Tmod4 to m-calpain–induced proteolysis in situ may be due to different conformations of Tmod1 and Tmod4 at the thin filament pointed ends, leading to differential accessibility of proteolytic epitopes; this may be mediated by sarcomeric Tmods’ differential affinities for striated muscle tropomyosins (Gokhin et al., 2010). Alternatively, the apparent lesser susceptibility of Tmod4 to m-calpain–induced proteolysis may be due to a greater abundance of Tmod4 than Tmod1 in TA and EDL myofibrils. To distinguish between these possibilities, we performed quantitative Western blotting of isolated skeletal muscle myofibrils prepared from TA and EDL muscles and determined the molar ratio of sarcomeric Tmods (Supplemental Figure S1). In three separate experiments, the ratio of Tmod4 to Tmod1 varied from 8.6 to 9.6 (Table 1), indicating that Tmod4 is almost an order of magnitude more abundant than Tmod1 on the thin filament pointed ends. In these experiments, the molar ratio of actin to total sarcomeric lengths, suggesting the existence of a ceiling on maximum thin filament length in vivo. Collectively these results delineate a novel mechanism contributing to skeletal muscle dysfunction in DMD in which calpain-mediated proteolysis of Tmods leads to actin subunit addition at thin filament pointed ends and increased thin filament lengths, and the types of muscles affected by this mechanism depend on the severity of DMD pathology.
Polyclonal antibody prepared against Tmod1 (Fowler et al., 1993). This discrepancy is most likely due to the fact that the immunoprecipitations were performed before the identification of Tmod4 (Almenar-Queralt et al., 1999), and the Tmod1 antibody failed to

Tmods varied from 187 to 208, corresponding to ~2 Tmods/pointed end (Table 1). This result is somewhat greater than the 1.2–1.6 Tmods/pointed end previously determined via quantitative immunoprecipitation of Tmods from isolated myofibrils using a rabbit polyclonal antibody prepared against Tmod1 (Fowler et al., 1993). This discrepancy is most likely due to the fact that the immunoprecipitations were performed before the identification of Tmod4 (Almenar-Queralt et al., 1999), and the Tmod1 antibody failed to
Completely immunoprecipitate Tmod4. We conclude that the apparent lesser susceptibility of Tmod4 to m-calpain-induced proteolysis in situ is due to the greater abundance of Tmod4 than Tmod1 in TA and EDL myofibrils. Moreover, these results collectively identify Tmod1 and Tmod4 as novel proteolytic substrates of m-calpain, implicating these Tmods as proteolytic targets of endogenous m-calpain in DMD-induced muscle degeneration.

Soleus muscles from mdx mice exhibit proteolysis of Tmod1 and increased thin filament lengths

DMD pathogenesis is characterized by sarcosomemal fragility, membrane rupture during muscle contraction, influx of extracellular Ca\(^{2+}\) into the muscle fiber interior, and hyperactivity of Ca\(^{2+}\)-dependent proteases, including calpains (Blake et al., 2002). It was previously shown that leg muscle from the mdx mouse model of DMD exhibits elevated concentrations of m-calpain (Spencer et al., 1995), although a systematic comparison of different muscle types was not performed. Therefore we used Western blots to measure m-calpain levels in TA, EDL, and soleus muscles from WT and mdx mice; these muscles were chosen because they reflect a diversity of muscle fiber recruitment levels and architectures (Burkholder et al., 1994). Results show that mdx soleus muscle, but not mdx TA or EDL muscle, exhibits its greater than twofold increase in m-calpain levels as compared with WT muscle (Figure 3, A and B). Thus, elevated m-calpain levels in DMD muscles appear to be primarily characteristic of heavily recruited, slow-twitch muscles such as the soleus.

To determine whether increased m-calpain levels in mdx soleus muscle are associated with proteolysis of sarcomeric Tmods in vivo, we immunostained longitudinal cryosections of TA and soleus muscles from postnecrotic (2-mo-old) WT and mdx mice for Tmod1 and Tmod4. In WT TA and soleus muscles, both Tmod1 and Tmod4 exhibited their well-established striated staining patterns of periodic doublets, corresponding to the pointed ends of phalloidin-stained thin filaments (Figures 4 and 5). Tmod1 and Tmod4 did not colocalize with m-calpain, which localizes to Z-line-flanking and M-line stripes (Supplemental Figure S2), possibly associated with the sarcoplasmic reticulum (SR; Gokhin and Fowler, 2011a). Of note, Tmod1 did not exhibit a striated staining pattern in mdx soleus muscle, instead showing a diffuse and punctate pattern consistent with proteolytic degradation (Figure 4B). By contrast, Tmod4 staining appeared to be unaffected in mdx soleus muscle, where it showed a pattern of striations at the thin filament pointed ends, indistinguishable from WT soleus muscle (Figure 5B). To confirm these results, we performed Western blots for Tmod1 and Tmod4. In agreement with the immunostaining data, Tmod1 showed ~40% decrease in protein levels in mdx soleus muscle, but Tmod1 levels were unchanged in mdx TA and EDL muscles (Figure 3, A and B). Similarly, Tmod4 levels were unchanged in mdx TA, EDL, and soleus muscles (Figure 3, A and B). Reverse transcription (RT)-PCR analysis showed identical Tmod1 and Tmod4 mRNA levels in WT and mdx muscles, indicating that reduced Tmod1 protein levels in mdx soleus muscle were not due to reduced transcription of the Tmod1 gene (Figure 3, E and F). We conclude that elevated m-calpain levels in mdx soleus muscle are associated with proteolytic degradation of Tmod1. This finding is consistent with the greater proteolytic sensitivity of myofibrillar Tmod1 as compared with Tmod4 in situ (Figure 1).

Pointed-end capping by Tmod1 restricts actin subunit association and dissociation from the pointed ends of actin filaments (Weber et al., 1994, 1999), and the extent of capping by Tmod1 is inversely related to thin filament length in sarcomeres of Drosophila and vertebrate cardiac muscles (Gregorio et al., 1995; Sussman et al., 1998; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001; Gokhin and Fowler, 2011b). Therefore we speculated that depletion of Tmod1 from mdx soleus muscle would allow actin subunit addition from the cytoplasmic pool onto the thin filament

| Experiment | Tmod1/gel sample (ng/µl) | Tmod4/gel sample (ng/µl) | Actin/gel sample (ng/µl) | Tmod4/Tmod1 (mol/mol) | Actin/sarcomeric Tmods (mol/mol) | Sarcomeric Tmods/pointed end |
|------------|--------------------------|--------------------------|--------------------------|------------------------|------------------------------|-------------------------------|
| 1          | 0.49 ± 0.03              | 4.25 ± 0.35              | 985 ± 45                 | 8.6                    | 208                          | 1.8                           |
| 2          | 0.36 ± 0.05              | 3.26 ± 0.22              | 722 ± 15                 | 8.8                    | 187                          | 2.1                           |
| 3          | 0.39 ± 0.03              | 3.84 ± 0.43              | 780 ± 68                 | 9.6                    | 193                          | 2.1                           |

*Amounts of sarcomeric Tmods were determined by quantitative Western blotting and densitometry of skeletal muscle myofibrils (1–8 µl of 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. SDs reflect three or four different myofibril volumes from different lanes on the same blot (Supplemental Figure S1).

*Amounts of actin were determined by densitometry of Coomassie blue-stained gels containing skeletal muscle myofibrils (1–8 µl of gel sample) electrophoresed alongside rabbit skeletal muscle actin standards (0.25–4 µg) on the same gel. SDs correspond to three or four different myofibril volumes from different lanes on the same gel (Supplemental Figure S1).

Numbers of Tmods/pointed end were calculated based on thin filament length of 1.11 µm and 13 actin subunits per 37 nm of thin filament (Fowler et al., 1993; Gokhin et al., 2010).

**TABLE 1: Stoichiometry of sarcomeric Tmods associated with thin filaments in mouse TA and EDL myofibrils.**
The usefulness of the mdx mouse as an animal model of DMD is restricted by its mild phenotype and normal lifespan, which are believed to be due to a host of mouse-specific compensatory responses, such as sustained postnatal up-regulation of utrophin (Law et al., 1994; Deconinck et al., 1997; Grady et al., 1997) and robust proliferative capacity of intramuscular stem cells to replace necrotic fibers (Sacco et al., 2010). Hence we examined Tmod isoform loss and measured thin filament lengths in mdx/mTR mice, a more severe animal model that recapitulates human DMD more accurately. In addition to lacking dystrophin, mdx/mTR mice lack the telomerase RNA component, leading to short telomeres and stem cell exhaustion during muscle regeneration (Sacco et al., 2010). Immunostaining of longitudinal cryosections of mdx/mTR TA muscles revealed disappearance of Tmod1 from the thin filament pointed ends, reflecting a more severe phenotype than in mdx TA muscle, where Tmod1 localization was normal (Figure 4A). Loss of Tmod1 from postnecrotic mdx/mTR TA muscle was due to DMD-induced proteolysis, and not absence of Tmod1 during myofibril assembly or muscle development, because TA muscles from prenecrotic (2-wk-old) mdx/mTR mice exhibited normal Tmod1 localization and correspondingly normal thin filament lengths (Supplemental Figure S3 and Supplemental Table S1). Tmod1 was also lost from the thin filament pointed ends in mdx/mTR soleus muscle, as in mdx soleus muscle (Figure 4B). Tmod4 showed normal localization in mdx/mTR TA muscle, similar to mdx TA muscle, although slight attenuation of the Z-line-flanking, SR-associated Tmod4 striations was observed pointed ends, leading to increased thin filament lengths. To test this, we performed DDecon analysis of line scans from fluorescence images to measure thin filament lengths from the breadth of the phalloidin signal and the distances of sarcomeric Tmods from the Z-line (Littlefield and Fowler, 2002; Gokhin and Fowler, 2013). Consistent with normal Tmod immunostaining and protein levels in mdx TA muscle, thin filament lengths in mdx TA muscle were indistinguishable from those in WT TA muscle (Table 2). On the other hand, thin filament lengths in mdx soleus muscle were ~12% greater than in WT soleus muscle (1.39 ± 0.10 vs. 1.24 ± 0.06 μm, respectively; Table 2), consistent with reduced Tmod1 levels in mdx soleus muscle. We conclude that calpain-mediated proteolysis of Tmod1 in mdx soleus muscle leads to thin filament elongation from pointed ends and increased thin filament lengths.

TA and soleus muscles from mdx/mTR mice exhibit proteolysis of Tmod1 and Tmod4 and increased thin filament lengths

FIGURE 3: Elevated levels of m-calpain are associated with decreased levels of sarcomeric Tmods in dystrophic muscles. (A) Western blots of homogenates of TA, EDL, and soleus muscles from 2-mo-old WT and mdx mice were probed using antibodies against m-calpain, Tmod1, and Tmod4. GAPDH was used as a loading control. (B) Quantification of Western blots. Error bars reflect mean ± SEM of n = 4 lanes/genotype within a single blot. (C) Western blots of homogenates of TA and soleus muscles from 2-mo-old WT and mdx/mTR mice were probed using antibodies against m-calpain, Tmod1, and Tmod4. GAPDH was used as a loading control. (D) Quantification of Western blots. Error bars reflect mean ± SEM of n = 3 lanes/genotype within a single blot. (E) RT-PCR of Tmod1 and Tmod4 mRNA transcripts from homogenates of TA, EDL, and soleus muscles from 2-mo-old WT and mdx mice. GAPDH was used as a loading control. (F) Quantification of RT-PCR. Error bars reflect mean ± SEM of n = 3 lanes/genotype within a single gel. *p < 0.05.
not necessarily result in longer thin filament lengths than disappearance of a single sarcomeric Tmod isoform, and thin filament length is not a simple inverse function of pointed-end capping by sarcomeric Tmods in skeletal muscle. Collectively our DDecon measurements show that calpain-mediated proteolysis of sarcomeric Tmods leads to increased thin filament lengths in mdx/mTR TA and soleus muscles.

The N-terminal M1M2M3 domain of nebulin is normally localized in dystrophic skeletal muscle

In skeletal muscle thin filaments, nebulin extends from its C-terminus in the Z-line to its N-terminal M1M2M3 domain at the end of the proximal segment, whereas the distal segment extends beyond the nebulin M1M2M3 domain and is capped by sarcomeric Tmods (Gokhin and Fowler, 2013). It is uncertain whether the increases in thin filament lengths in dystrophic muscle are due to longer distal segments, longer proximal segments, or a combination of both. To distinguish among these possibilities, we immunostained longitudinal cryosections of TA and soleus muscles from WT, mdx, and mdx/mTR mice for the nebulin M1M2M3 domain and measured the distances of the nebulin M1M2M3 domain from the Z-line. In TA and soleus muscles of all three genotypes, the nebulin M1M2M3 domain localized in striations slightly proximal to the pointed ends of the thin filaments (Figure 6), as expected (Castillo et al., 2009; Gokhin et al., 2010, 2012; Gokhin and Fowler, 2013). The nebulin M1M2M3 striations were ~0.92–0.94 μm from the Z-line in both TA and soleus muscles of all three genotypes, and no significant differences among the genotypes were detected (Table 2). Therefore the lengths of the nebulin-coated proximal segments are unchanged in mdx and mdx/mTR muscles, and the overall increases in thin filament lengths observed in these muscles are driven entirely by increases in the lengths of the nebulin-free, Tmod-capped distal segments.

SR-associated actin cytoskeletal proteins are normally localized in dystrophic skeletal muscle

Tmod3, an SR-associated Tmod isoform, has been shown to structurally compensate for the absence of Tmod1 in Tmod1−/− skeletal muscle by dissociating from its SR compartment and relocating to the thin filament pointed ends, thereby maintaining normal thin filament lengths (Gokhin et al., 2010; Gokhin and Fowler, 2011a). To test whether SR-associated Tmod3 may also compensate for depletion of Tmod1 and/or Tmod4 in dystrophic muscles via a similar relocation-based mechanism, we immunostained longitudinal cryosections of TA and soleus muscles from WT, mdx, and mdx/mTR mice for Tmod3. Surprisingly, Tmod3 localized to Z-line-flanking and M-line stripes (Figure 7A) in TA muscles of all three genotypes, indicative of Tmod3’s normal localization in the SR (Gokhin and Fowler, 2011a). In soleus muscles of all three genotypes, Tmod3 also localized to Z-line-flanking stripes, with fainter M-line localization.
Examination of highly stretched myofibrils in all three genotypes did not reveal any Tmod3 associated with the thin filament pointed ends that might have been obscured by the bright Tmod3 fluorescence at the M-line (unpublished data). Thus, in contrast to Tmod1−/− skeletal muscle, Tmod3 fails to cap Tmod1-free thin filament pointed ends and compensate for depletion of sarcomeric Tmods in dystrophic muscle.

Tmod3 caps the pointed ends of cytoplasmic γ-actin (γcyto-actin) filaments and interacts with nonmuscle tropomyosins and small ankyrin 1.5 to mechanically stabilize the SR membrane (Gokhin and Fowler, 2011a). Dystrophic muscles exhibit dramatic up-regulation of γcyto-actin, presumably as a compensatory fortification response to sarcolemmal fragility (Hanft et al., 2006, 2007). On the basis of these observations, we speculated that depletion of sarcomeric Tmods from dystrophic muscle would result in incorporation of excess γcyto-actin into the longer thin filaments, as observed in myofibrils from γcyto-actin–overexpressing muscle (Jaeger et al., 2009).

To test this, we immunostained longitudinal cryosections of TA and soleus muscles from WT, mdx, and mdx/mTR mice for γcyto-actin. In TA muscles of all three genotypes, γcyto-actin localized to Z-line-flanking and M-line stripes (Figure 8A), consistent with its previously observed localization in the SR (Gokhin and Fowler, 2011a). In soleus muscles of all three genotypes, γcyto-actin localized to a wide I-band stripe and lacked

TABLE 2: Thin filament lengths in 2-mo-old mice determined by DDecon analysis of fluorescence images.
M-line localization (Figure 8B), revealing the presence of different γcyto-actin architectures in TA versus soleus muscles, which remain unaltered in mdx and mdx/mTR mice. Of note, γcyto-actin staining was markedly brighter in TA and soleus muscles from mdx and mdx/mTR mice (Figure 8), consistent with increased γcyto-actin levels in dystrophic muscles (Hanft et al., 2006, 2007). Both increased γcyto-actin and correspondingly increased Tmod3 levels were confirmed by Western blotting (Supplemental Figure S4). However, γcyto-actin was not enriched at the Z-line or H-zone periphery in mdx or mdx/mTR muscles (Figure 8), indicating no incorporation of γcyto-actin into the barbed or pointed ends of the thin filaments, respectively. We conclude that depletion of sarcomeric Tmods from dystrophic skeletal muscle does not enable incorporation of γcyto-actin into the thin filaments.

DISCUSSION
We identified calpain-mediated proteolysis of sarcomeric Tmods and resultant elongation of thin filaments from their pointed ends in skeletal muscles from murine models of DMD. Two factors influence this mechanism: 1) disease severity (low vs. high in mdx vs. mdx/mTR muscles, respectively), and 2) muscle recruitment and use (infrequent vs. frequent in TA vs. soleus muscles, respectively). By systematically analyzing Tmod isoform localizations and levels, we showed that mdx soleus and mdx/mTR TA muscles lose only Tmod1 via calpain-mediated proteolysis, whereas mdx/mTR soleus muscle loses both Tmod1 and Tmod4 (summarized in Figure 9). Thus dystrophic muscle provides a useful experimental system in which to study thin filament length regulation by sarcomeric Tmods and suggests that misspecification of thin filament length may contribute to muscle pathology in DMD. Note that the observed relationship between elevated m-calpain levels and Tmod proteolysis is correlative and not necessarily causative. Other proteolytic pathways may also contribute to Tmod proteolysis in DMD.

Implications for thin filament length regulation in skeletal muscle
In Drosophila and vertebrate cardiac muscles, thin filaments lack nebulin, and thin filament lengths are inversely related to the extent of pointed-end capping by sarcomeric Tmods, with greater capping yielding shorter lengths and reduced capping yielding longer lengths (Gregorio et al., 1995; Sussman et al., 1998; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001; Gokhin and Fowler, 2011b). By contrast, in skeletal muscle, thin filaments contain nebulin and are divided into two structurally distinct segments: 1) a constant-length, nebulin-coated proximal segment anchored at the Z-line, and 2) a variable-length, nebulin-free distal segment capped by sarcomeric Tmods at the H-zone periphery (Gokhin and Fowler, 2013). This study identifies mdx and mdx/mTR mice as the first in vivo models of Tmod isoform depletion leading to thin filament elongation in skeletal muscle, demonstrating that the lengths of the nebulin-free distal segments of skeletal muscle thin filaments are regulated by sarcomeric Tmods via a similar inverse manner as the nebulin-free thin filaments in Drosophila and vertebrate cardiac muscles. A corollary of this conclusion is that the nebulin-free thin filaments in Drosophila and vertebrate cardiac muscles are effectively “ultralong distal segments” lacking nebulin-coated proximal segments (Gokhin and Fowler, 2013).

Intriguingly, comparison of WT, mdx, and mdx/mTR soleus muscles reveals that graded losses of sarcomeric Tmod isoforms do not result in correspondingly graded increases in thin filament lengths. Namely, mdx soleus (lacking Tmod1) and mdx/mTR soleus (lacking both Tmod1 and Tmod4) have statistically indistinguishable thin filament lengths of 1.28 ± 0.09 and 1.29 ± 0.06 μm, respectively. The additional loss of Tmod4 from mdx/mTR soleus without a further increase in thin filament length, as compared with mdx soleus, where only Tmod1 is lost, also suggests unique functions for Tmod1 and Tmod4 in sarcomeres. Whereas Tmod1 is clearly important for length regulation, Tmod4 appears to be less important and may instead play a role in regulating actomyosin contractility (see later discussion). Previous studies using quantitative immunoprecipitation with pan-Tmod antibodies led to the conclusion that there are
FIGURE 7: Tmod3 exhibits normal, SR-associated localization in mdx and mdx/mTR muscles. (A, B) Longitudinal cryosections of (A) TA and (B) soleus muscles from 2-mo-old WT, mdx, and mdx/mTR mice were immunostained for Tmod3 and α-actinin and phalloidin-stained for F-actin. Bars, 1 μm.

1.2–1.6 Tmods/thin filament in rat psoas muscles (Fowler et al., 1993). However, based on the quantitative Western blotting experiments presented here, Tmod1 is an order of magnitude less abundant than Tmod4 in sarcomeres, and ~2 Tmods cap each thin filament in mouse skeletal muscles. Nevertheless, loss of Tmod1 in the mdx soleus can still result in an increase in average thin filament length, even when the protein levels and immunostaining pattern of the more abundant Tmod4 isoform remain unchanged. Calculations of sarcomeric Tmod concentrations based on 2 Tmods/pointed end (this study), a mouse skeletal muscle α-actin concentration of 893 μM (Hanft et al., 2006), and subcellular fractionation of mouse skeletal muscle showing ~50% of Tmod1 and Tmod4 associated with myofibrils (Gokhin and Fowler, 2011a) yield a total sarcomeric Tmod concentration of ~9.2 μM and a Tmod1 concentration of just 0.92 μM. Therefore, even when ~50% of the Tmod1 is degraded in the mdx soleus, total Tmod is reduced by just 5%. Thus it is surprising that the more abundant Tmod4 fails to occupy the vacant ends formerly occupied by Tmod1 in the mdx soleus. This is not because the cytosolic concentration of sarcomeric Tmods (~1.84 μM) is below the K_d of Tmod1 or Tmod4 binding to tropomyosin-coated actin filaments (~10 pM; Almenar-Queralt et al., 1999; Weber et al., 1999). A consequence of this is that the cytosolic subpopulations of Tmod1 and Tmod4 are unlikely to freely exchange with the thin filament pointed-end populations. We speculate that, instead, thin filament-associated Tmod4 dynamically shuttles among adjacent pointed ends to regulate thin filament lengths within a sarcomere, similar to a proposed mechanism of Tmod1 dynamics in cardiac myocytes (Littlefield et al., 2001). Therefore, when Tmod1 is depleted, a reduced ratio of total Tmod to pointed ends can lead to actin subunit addition and longer thin filaments in skeletal muscle, similar to observations in cardiac and Drosophila muscle (Gregorio et al., 1995; Sussman et al., 1998; Littlefield et al., 2001; Mardahl-Dumesnil and Fowler, 2001; Bai et al., 2007).

Our findings further show that uniformity of thin filament lengths is well regulated in the mdx/mTR soleus, even when both Tmod1 and Tmod4 are depleted and thin filament lengths increase by ~12%. This implies the existence of additional structural and/or regulatory constraints that restrict uncontrolled elongation of thin filaments from their pointed ends. Two such potential constraints are 1) limited availability of actin monomers in the cytosol (Shimizu and Obi, 1986) and 2) pointed ends interacting with as-yet-unidentified M-line or H-zone constituents (Gokhin and Fowler, 2013). It is noteworthy that mdx/mTR soleus muscle also exhibits occasional patches of myofilament degeneration and disorganization, and we cannot exclude the possibility that any “overly long” thin filaments that appear in the mdx/mTR soleus may be transient or unstable, subsequently disassembling via ADF/cofilin-mediated actin depolymerization (Nagaoka et al., 1995; Ono and Ono, 2002; Miyauchi-Nomura et al., 2012) or some other disassembly mechanism. Such disassembly would be reminiscent of—but not identical to—observations in cultured cardiomyocytes, in which antibody inhibition of Tmod1/tropomyosin binding leads to thin filament disassembly (Mudry et al., 2003).

In Tmod1<sup>−/−</sup> mouse skeletal muscle, thin filament lengths are normal because Tmod3 structurally compensates for the absence of Tmod1 by dissociating from its SR-associated compartment and redistributing to the thin filament pointed ends (Gokhin et al., 2010; Gokhin and Fowler, 2011a). However, Tmod3 fails to compensate for depletion of Tmod1 (and Tmod4) in dystrophic muscle. The most likely explanation for this discrepancy is that Tmod3 can only assemble in the complete absence of Tmod1 during muscle development, prior to Tmod3 associating with its binding partners in the SR. It appears that, in Tmod1<sup>−/−</sup> muscle, absence of Tmod1 eliminates the competition between Tmod1 and Tmod3 for the striated muscle tropomyosins at the pointed ends (Gokhin et al., 2010), thereby enabling Tmod3 to aberrantly assemble onto the thin filament pointed ends. By contrast, in adult mdx and mdx/mTR muscle, Tmod3 is already associated with the pointed ends of γ<sub>SR</sub>-actin filaments in the SR-associated cytoskeletal network before the onset of muscle damage (Gokhin and Fowler, 2011a). Thus, even as contraction-induced sarcolemmal rupture leads to Ca<sup>2+</sup> influx, calpain activation, and proteolytic depletion of sarcomeric Tmods in mdx and mdx/mTR muscles, Tmod3’s interactions with its SR-associated binding partners...
Tropomodulins in dystrophic muscle and human nemaline myopathy patients have shown that thin filament length–tension curve, especially given the experimental variability, is too small to induce measurable changes in the sarcomere length–tension curve (Granzier et al., 1991; Gokhin et al., 2009; Ottenheijm et al., 2009, 2010). Such thin filament length changes are markedly greater than the thin filament length changes of as much as 0.30 μm may be required to significantly alter the sarcomere length–tension curve (Granzier et al., 1991; Gokhin et al., 2009; Ottenheijm et al., 2009, 2010). These observations, in conjunction with our findings that m-calpain proteolyzes actomyosin cross-bridges in mdx and mdx/mTR muscles, collectively imply that the biophysical mechanism is not understood, Tmod1's interactions with F-actin and/or terminal tropomyosins at the pointed end may mediate a long-range conformational change in the thin filament that facilitates cross-bridge recruitment. Tmod4 may also play its own as-yet-undetermined role in regulating thin filament activation and/or cross-bridge recruitment, distinct from Tmod1. Thus we might expect sarcomeric Tmod-depleted fibers from mdx and mdx/mTR muscles to also exhibit attenuated thin filament activation and impaired cross-bridge recruitment. Future studies will be required to explore this prediction experimentally using skinning fiber mechanics with mdx/mTR TA muscles, as soleus muscles are unsuitable for skinned fiber mechanics. However, based on this prediction, it is tempting to speculate that increasing actomyosin contractility via pharmacological interventions (e.g., troponin activators) may ameliorate muscle weakness in DMD. Such a strategy should be approached with caution, because increased actomyosin contractility has the potential to exacerbate sarcomemmal damage in dystrophic muscle, thereby canceling out any potentially beneficial effect of the increased actomyosin contractility. Preclinical studies using dystrophic mouse models are underway in order to resolve this dilemma (Miciak et al., 2013).

Although it has been established that calpain-mediated proteolysis contributes to skeletal muscle degeneration in DMD (Spencer and Tidball, 1992; Spencer et al., 1995; Tidball and Spencer, 2000), identifying the molecular mechanisms of calpain function in vivo has been challenging due to the paucity of confirmed calpain substrates and the widely variable amino acid sequences surrounding calpain cleavage sites (Carafoli and Molinari, 1998; Goll et al., 2003). Calpains generally favor cytoskeletal proteins, including F-actin–associated proteins, such as spectrin (reviewed in Czogalla and Sikorski, 2005), filamin (Wencel-Drake et al., 1991), and cortactin (Perrin et al., 2006). Calpain also proteolyzes utrophin (Couder-Fruh and Briguet, 2006), a dystrophin homologue and actin-binding protein that compensates for dystrophin deficiency in mdx mouse muscle but not human DMD (Law et al., 1994; Deconinck et al., 1997; Grady et al., 1997; Galkin et al., 2002; Rybakova et al., 2002, 2006). These observations, in conjunction with our findings that m-calpain proteolyzes sarcomeric Tmod3 in mdx and mdx/mTR muscles, collectively may render it unable to translocate to the thin filament pointed ends. Indeed, stable association of Tmod3 with the SR is supported by its resistance to proteolysis in mdx and mdx/mTR muscles.

Implications for DMD pathology

Thin filament elongation due to depletion of sarcomeric Tmods in mdx and mdx/mTR muscles indicates that secondary, myofibril-intrinsic changes are likely to contribute to muscle weakness in DMD, occurring in conjunction with the primary changes directly resulting from dystrophin deficiency (i.e., sarcolemmal fragility and impaired lateral force transmission). First, sliding filament theory predicts that thin filament elongation due to depletion of sarcomeric Tmods will result in a widening and rightward shift in the sarcomere length–tension relationship and increased optimum sarcomere length for force production in mdx and mdx/mTR muscles (Granzier et al., 1991; Gokhin and Fowler, 2013). However, physiological experiments have shown no such changes in mdx muscles (Coulton et al., 1988). The most likely explanation for this discrepancy is that the thin filament length changes observed in mdx and mdx/mTR muscles are too small to induce measurable changes in the sarcomere length–tension curve, especially given the experimental variability associated with conventional physiological assays. Indeed, physiological analyses of skeletal muscles from perch fish, nebulin−/− mice, and human nemaline myopathy patients have shown that thin filament length changes of as much as 0.30 μm may be required to significantly alter the sarcomere length–tension curve (Granzier et al., 1991; Gokhin et al., 2009; Ottenheijm et al., 2009, 2010).

Second, depletion of sarcomeric Tmods may inhibit the formation of productive actomyosin cross-bridges in mdx and mdx/mTR muscles. Skinned fiber mechanics and x-ray diffraction studies of Tmod1−/− muscles reveal reduced tropomyosin strand movement and actomyosin cross-bridge recruitment during thin filament activation (Ochala et al., 2014). Although the biophysical mechanism is not understood, Tmod1's interactions with F-actin and/or terminal tropomyosins at the pointed end may mediate a long-range conformational change in the thin filament that facilitates cross-bridge recruitment. Tmod4 may also play its own as-yet-undetermined role in regulating thin filament activation and/or cross-bridge recruitment, distinct from Tmod1. Thus we might expect sarcomeric Tmod-depleted fibers from mdx and mdx/mTR muscles to also exhibit attenuated thin filament activation and impaired cross-bridge recruitment. Future studies will be required to explore this prediction experimentally using skinning fiber mechanics with mdx/mTR TA muscles, as soleus muscles are unsuitable for skinned fiber mechanics. However, based on this prediction, it is tempting to speculate that increasing actomyosin contractility via pharmacological interventions (e.g., troponin activators) may ameliorate muscle weakness in DMD. Such a strategy should be approached with caution, because increased actomyosin contractility has the potential to exacerbate sarcolemmal damage in dystrophic muscle, thereby canceling out any potentially beneficial effect of the increased actomyosin contractility. Preclinical studies using dystrophic mouse models are underway in order to resolve this dilemma (Miciak et al., 2013).
suggest that F-actin remodeling is an important contributor to muscle dysfunction in DMD. Our findings also implicate Tmods as potential calpain cleavage targets in other pathological events characterized by heightened calpain activity, such as traumatic brain injury, stroke, and myocardial infarction (Carafoli and Molinari, 1998; Goll et al., 2003). Future studies will seek to examine such Tmod proteolysis and F-actin remodeling in human patients.

MATERIALS AND METHODS

Experimental animals

WT and mdx mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mdx/mTR mice have been described previously (Sacco et al., 2010). The mdx/mTR mice were bred for two generations to minimize telomere length while maintaining mouse viability, as described previously (Sacco et al., 2010; Mourkioti et al., 2013). Because DMD is an X-linked disorder, experiments were restricted to male mice. Mice were either 2 wk or 2 mo old at the time of killing, corresponding to time points before or after the onset of muscle necrosis, respectively (Anderson et al., 1988; Coulton et al., 1988). Mice were killed by isoflurane inhalation followed by cervical dislocation. All procedures were performed in accordance with animal care guidelines enforced by the Institutional Animal Care and Use Committee at the Scripps Research Institute.

Antibodies

Primary antibodies and dilutions were as follows: affinity-purified rabbit polyclonal anti-human Tmod1 (R1749bl3c, 3.1 μg/ml for cryosections, 0.31 μg/ml for isolated myofibrils; Gokhin et al., 2010), rabbit polyclonal antisera to residues 340–359 of a human Tmod1 peptide (PA2211, 1:5000 for Western blots; Gokhin et al., 2012), rabbit polyclonal antisera to chicken Tmod4 preadsorbed by passage through a Tmod1 Sepharose column (R3577bl3c, 1:25 for cryosections, 1:250 for isolated myofibrils, 1:2500 for Western blots; 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), affinity-purified rabbit polyclonal anti-nebulin M1M2M3 domain (R1357L, 9.3 μg/ml for cryosections; a gift from Carol C. Gregorio, University of Arizona, Tucson, AZ), affinity-purified rabbit polyclonal anti-γcyto-actin (7577, 0.9 μg/ml for cryosections, 0.09 μg/ml for Western blots; a gift from James M. Ervasti, University of Minnesota, Minneapolis, MN), mouse monoclonal anti–m-calpain (107-82, 1:50 for cryosections, 1:1000 for Western blots; Thermo Fisher Scientific, Waltham, MA), mouse monoclonal anti–α-actinin (EA53, 1:100 for cryosections, 1:1000 for isolated myofibrils, 1:10,000 for Western blots; Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-actin (C4, 1:10,000 for Western blots; EMD Millipore, Billerica, MA), and mouse monoclonal anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1D4, 1:5000 for Western blots; Novus Biologicals, Littleton, CO). Secondary antibodies and dilutions were as follows: Alexa Fluor 488–conjugated goat anti-rabbit immunoglobulin G (iG; 1:200 for cryosections, 1:1000 for isolated myofibrils; Life Technologies, Carlsbad, CA), Alexa Fluor 647–conjugated goat anti-mouse IgG (1:200 for cryosections, 1:1000 for isolated myofibrils; Life Technologies), 800CW-conjugated goat anti-mouse 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).

Preparation and immunostaining of isolated myofibrils

Myofibril preparation procedures were modified from those described previously (Knight and Trinick, 1982; Castillo et al., 2009). Briefly, two TA and two EDL muscles were excised from mouse hindlimbs, stretched, pinned to cork, and relaxed overnight in

![FIGURE 9: Model of pointed-end capping by sarcomeric Tmods and thin filament elongation in dystrophic skeletal muscle. TA and soleus muscles differ by their fiber type distributions, recruitment levels, and thin filament (distal segment) lengths. In mdx mice (mild dystrophy), soleus muscle exhibits depletion of Tmod1 and thin filament elongation. In mdx/mTR mice (severe dystrophy), TA muscle exhibits depletion of Tmod1, soleus muscle exhibits depletion of both Tmod1 and Tmod4, and both TA and soleus exhibit thin filament elongation. For simplicity, one actin strand, one nebulin molecule, one tropomyosin polymer, and one Tmod molecule are depicted for each thin filament. Also for simplicity, equal proportions of Tmod1 and Tmod4 are depicted for each WT sarcomere.](image-url)
ice-cold relaxing buffer (100 mM NaCl, 2 mM KCl, 2 mM MgCl₂, 6 mM K₃PO₄, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1% glucose, pH 7.0) supplemented with protease inhibitor cocktail (1:1000; Life Technologies). TA and EDL tissues were pooled due to their functional similarity and nearly identical fiber type distributions (Burkholder et al., 1994). Muscles were then transferred to ice-cold rigor buffer (100 mM KCl, 10 mM K₃PO₄, 2 mM MgCl₂, 1 mM EGTA, pH 7.0) for 10 min, placed in a conical tube containing 5 ml of rigor buffer and protease inhibitor cocktail (1:1000; Life Technologies), and homogenized with a Tekmar Tissumizer at 70% of maximum speed for 90 s. The homogenate was then centrifuged at 1000 g for 5 min, the supernatant was decanted, and myofibrils were resuspended in ice-cold rigor buffer. Centrifugation/resuspension was repeated three times to clean the myofibril suspension (~10:1 buffer:myofibrils after washing), which was then divided into aliquots, to which 10 mM CaCl₂ and increasing concentrations of m-calpain (BioVendor, Asheville, NC) ± 10 μg/ml calpeptin (Enzo Life Sciences, Farmingdale, NY) were added. Myofibril suspensions were incubated for 1 h at room temperature, dropped onto poly-L-lysine-coated microscope slides (Polysciences, Warrington, PA), and allowed to adhere for 30 min. Adherent myofibrils were then fixed for 30 min in 4% paraformaldehyde (PFA) in rigor buffer, washed for 5 min in phosphate-buffered saline (PBS) plus 0.1% Triton X-100 (PBST), and then blocked for 1 h in 4% bovine serum albumin (BSA) plus 1% goat serum in PBST at room temperature. Myofibrils were then labeled with primary antibodies diluted in blocking buffer for 1 h at room temperature, washed for 5 min in PBST, and labeled with a fluorophore-conjugated secondary antibody mixture in blocking buffer for 1 h at room temperature. The secondary antibody mixture was supplemented with rhodamine-phalloidin (1:500; Life Technologies) to stain F-actin. Myofibrils were then washed again in PBST, preserved in Gel Mount aqueous mounting medium (Sigma-Aldrich), and coverslipped.

**Preparation and immunostaining of muscle tissue cryosections**

Mouse hindlimbs were pinned to cork, immersed in ice-cold relaxing buffer, relaxed for 24 h at 4°C, and then fixed in 4% PFA in relaxing buffer for 24 h at 4°C. TA and soleus muscles were then excised, cryoprotected in 30% sucrose in PBS for 4 h, embedded in Tissue-Tek OCT Compound (Sakura Finetek USA, Torrance, CA), and frozen on a metal block chilled in liquid N₂. Muscles were divided into 12-μm-thick cryosections, mounted on slides, and stored at −20°C until use. Sections were washed in PBST, permeabilized for 15 min in PBS plus 0.3% Triton X-100, and blocked for 2 h in 4% BSA plus 1% goat serum in PBST. Tissue were labeled with primary antibodies diluted in blocking buffer overnight at 4°C, washed three times in PBST, and then labeled with a fluorophore-conjugated secondary antibody mixture in blocking buffer for 2 h at room temperature. The secondary antibody mixture was supplemented with rhodamine-phalloidin (1:100; Life Technologies) to stain F-actin. Tissue were then washed three times in PBST, preserved in Gel Mount aqueous mounting medium (Sigma-Aldrich), and coverslipped.

**Confocal imaging**

Images of single optical sections were collected on a Bio-Rad Radiance 2100 laser-scanning confocal microscope (Bio-Rad, Hercules, CA) mounted on a Nikon TE2000-U microscope (Nikon, Melville, NY) using a 100x (1.4 numerical aperture) Plan-Apochromat oil-objective lens at room temperature (zoom = 3). Bio-Rad LaserSharp 2000 software was used during image collection. Images were processed with Photoshop (Adobe, San Jose, CA), and image figures were constructed in Adobe Illustrator. Supplemental Figure S5 shows diagrams of sarcomeres indicating the expected locations of the various fluorescently labeled proteins in Figures 4–8.

**Thin filament length measurements**

DDecon was used to measure distances of Tmod1 and Tmod4 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; Littlefield and Fowler, 2002). Analysis was performed using a DDecon plug-in originally developed for ImageJ (National Institutes of Health, Bethesda, MD) by Ryan S. Littlefield (University of Washington, Seattle, WA) and modified by Zhen Ren (The Scripps Research Institute, La Jolla, CA). This plug-in is available for public download at http://www.scripps.edu/fowler/. The DDecon plug-in generates the best fit of a model intensity distribution function for a given thin filament component (Tmod or F-actin) to an experimental one-dimensional myofibril fluorescence intensity profile (line scan) of three thin filament arrays obtained for each fluorescent probe (anti-Tmod or phalloidin; Littlefield and Fowler, 2002). Model fitting of background-corrected line scans was optimized by an iterative fitting procedure that minimizes the error between the observed line-scan intensities and the modeled intensities, using a multivariate line-fitting algorithm, as described previously (Littlefield and Fowler, 2002). Distances were calculated by converting pixel sizes into micrometers based on each image's magnification factor (25.6 pixels/μm).

**Western blotting**

For Western blotting of whole-muscle homogenates, TA, EDL, and soleus 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; Life Technologies). For Western blotting of isolated myofibrils, myofibril suspensions were prepared as described under Preparation and immunostaining of isolated myofibrils. 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). 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 Western transfer efficiencies of endogenous versus recombinant purified Tmods. (The Tmod4−/− mouse was made from mouse embryonic stem cells with a lacZ/neo insertion in exon 2 of the Tmod4 gene, and its phenotype will be described in detail elsewhere.)

Muscles, myofibrils, and purified protein preparations were solubilized in an equal volume of 2X SDS sample buffer and boiled for 5 min. Proteins were separated via SDS–PAGE on 4–20% Tris-glycine gradient minigels for 1 h at 200 V and transferred to nitrocellulose (pore size, 0.2 μm), as described previously (Gokhin et al., 2010). Blots were stained with 0.2% Ponceau S in 3% trichloroacetic acid to verify protein transfer, blocked for 2 h in 4% BSA plus 1% goat serum in PBS at room temperature, and then incubated in primary antibodies diluted in Blitz buffer (4% BSA, 10 mM NaHPO₄, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.4) overnight at 4°C. After washing in PBST, blots were incubated in secondary antibodies diluted in Blitz for 1 h at room temperature. After washing again in PBST, bands were visualized using a LI-COR Odyssey infrared imaging system and densitometrically quantified using ImageJ (with normalization to GAPDH, where appropriate).
RT-PCR
Total RNA was extracted from frozen TA, EDL, and soleus muscles using TRIzol reagent (Qiagen, Valencia, CA) and reverse transcribed using oligo(dT) primers and M-MLV reverse transcriptase (Life Technologies), according to the manufacturer’s instructions. Primers were as follows: Tmd1 sense, 5′-CAACCCCATGATGAGCAAC-3′; Tmd1 antisense, 5′-CAGCGTAAAGACCCTGCCAG-3′; Tmd4 sense, 5′-GATGCCGAGCTGAGATGGAGATG-3′; Tmd4 antisense, 5′-TCTCTT- CCTTTGCTGACGACG-3′; GAPDH sense, 5′-GGGATCTTGGGGCTACACT-3′; and GAPDH antisense, 5′-GAGGAAATGCGACCCGGCG-3′. PCR was then performed using a Bio-Rad T100 thermal cycler. An initial hold at 95°C for 1 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 20 s. After a final hold at 72°C for 5 min, reaction products were electrophoresed on agarose gels, stained with EtBr, and visualized using a Multilmage Light Cabinet (Alpha Innotech, San Leandro, CA). Tmd1 and Tmd4 band intensities were densitometrically quantified using ImageJ with normalization to GAPDH.

In vitro proteolysis
Recombinant human Tmd1 and mouse Tmd4 were purified as described previously (Yamashiro et al., 2010). Increasing concentrations of m-calpain were mixed with 3 μg of Tmd1 or Tmd4 in a final volume of 10 μl, and mixtures were then incubated for 1 h at room temperature, solubilized in an equal volume of 2x SDS sample buffer, and boiled for 5 min. Proteins were separated via SDS–PAGE on 4–20% Tris-glycine gradient minigels for 1 h at 200 V. Gels were then stained with Coomassie blue for 10 min, destained overnight, scanned at 600 dpi, and densitometrically quantified using ImageJ.

Statistics
Data are presented as either mean ± SD or mean ± SEM, where appropriate. Differences between two groups were detected using Student’s t test. Differences between three groups were detected using one-way analysis of variance with post hoc Fisher’s PLSD tests. Statistical significance was defined as p < 0.05. Statistical analysis was performed in Excel (Microsoft, Redmond, WA).

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REFERENCES
Alderton JM, Steinhardt RA (2000). Calcium influx through calcium leak channels is responsible for the elevated levels of calcium-dependent proteolysis in dystrophic myotubes. J Biol Chem 275, 9452–9460.
Almenar-Queralt A, Lee A, Conley CA, Ribas de Pouplana I, Fowler VM (1999). Identification of a novel tropomodulin isoform, skeletal tropomodulin, that caps actin filament pointed ends in fast skeletal muscle. J Biol Chem 274, 28466–28475.
Anderson JE, Bressler BH, Ovalle WK (1988). Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. J Muscle Res Cell Motil 9, 499–515.
Bai J, Hartwig JH, Perrimon N (2007). SALS, a WH2-domain-containing protein, promotes sarcomeric actin filament elongation from pointed ends during Drosophila muscle growth. Dev Cell 13, 828–842.
Blake DJ, Weir A, Newey SE, Davies KE (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev 82, 291–329.
Briquet A et al. (2008). Effect of calpain and proteasome inhibition on Ca2+-dependent proteolysis and muscle histopathology in the mdx mouse. FASEB J 22, 4190–4200.
Brinksteder TJ, Fingado B, Baron S, Lieber RL (1994). Relationship between muscle fiber types and sizes and muscle architectural properties in the mouse hindlimb. J Morphol 221, 177–190.
Carafoli E, Molinari M (1998). Calpain: a protease in search of a function. Biochem Biophys Res Commun 247, 193–202.
Castillo A, Nowak R, Littlefield KP, Fowler VM, Littlefield RS (2009). A nebulin ruler does not dictate thin filament lengths. Biophys J 96, 1856–1865.
Childers MK, Bogan JR, Bogan DJ, Greiner H, Holder M, Grange RW, Kornegay JN (2011). Chronic administration of a leupeptin-derived calpain inhibitor fails to ameliorate severe muscle pathology in a canine model of Duchenne muscular dystrophy. Front Pharmacol 2, 89.
Combaret L, Taillandier D, Voisin L, Samuels SE, Boespflug-Tanguy O, Autre D (1996). No alteration in gene expression of components of the ubiquitin-proteasome proteolytic pathway in dystrophin-deficient muscles. FEBS Lett 393, 292–296.
Coulton GR, Curtin NA, Morgan JE, Partridge TA (1988). The mdx mouse skeletal muscle myopathy: II. contractile properties. Neuromuscul Appl Neurobiol 14, 299–314.
Courdier-Frihr I, Briquet A (2006). Utrophin is a calpain substrate in muscle cells. Muscle Nerve 33, 753–759.
Czogalla A, Skorski AF (2005). Spectrin and calpain: a “target” and a “sniper” in the pathology of neuronal cells. Cell Mol Life Sci 62, 1913–1924.
Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson JG, Tinsley JM, Davies KE (1997). Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell 90, 717–727.
Fowler VM, Sussmann MA, Miller PG, Flucher BE, Daniels MF (1993). Tropomodulin is associated with the free (pointed) ends of the thin filaments in rat skeletal muscle. J Cell Biol 120, 411–420.
Gailly P, De Backer F, Van Schoor M, Gillis JM (2007). In situ measurements of calpain activity in isolated muscle fibers from normal and dystrophin-lacking mdx mice. J Physiol 582, 1261–1275.
Galkin VE, Orlova A, VanLoock MS, Rybakova IN, Ervasti JM, Egelman EH (2002). The utrophin actin-binding domain binds F-actin in two different modes: implications for the spectrin superfamily of proteins. J Cell Biol 157, 243–251.
Gokhin DS, Bang ML, Zhang J, Lieber RL (2009). In vitro proteolysis of the utrophin actin-binding domain inclusions from mdx skeletal muscle. Muscle Nerve 34, 291–300.
Gokhin DS, Fowler VM (2011). Cytoplasmic gamma-actin and tropomodulin isoforms link to the sarcoplasmic reticulum in skeletal muscle fibers. J Cell Biol 194, 105–120.
Gokhin DS, Fowler VM (2011b). Tropomodulin capping of actin filaments in striated muscle development and physiology. J Biomed Biotechnol 2011, 103069.
Gokhin DS, Fowler VM (2013). A two-segment model for thin filament architecture in skeletal muscle. Nat Rev Mol Cell Biol 14, 113–119.
Gokhin DS, Kim NE, Lewis SA, Hoenecke HR, D’Lima DD, Fowler VM (2012). Thin-filament length correlates with fiber type in human skeletal muscle. Am J Physiol Cell Physiol 302, C555–C565.
Gokhin DS, Lewis RA, McKeown CR, Nowak RB, Kim NE, Littlefield RS, Lieber RL, Fowler VM (2010). Tropomodulin isoforms regulate thin filament pointed-end capping and skeletal muscle physiology. J Cell Biol 189, 95–109.
Goll DE, Thompson VF, Li H, Wei W, Cong J (2003). The calpain system. Physiol Rev 83, 731–801.
Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR (1997). Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell 90, 729–738.
Grazner HL, Akster HA, Ter Keurs HE (1991). Effect of thin filament length on the force-sarcomere length relationship of skeletal muscle. Am J Physiol 260, C1060–C1070.
Gregorio CC, Weber A, Bondad M, Pennise CR, Fowler VM (1995). Requirement of pointed-end capping by tropomodulin to maintain actin filament length in embryonic chick cardiac myocytes. Nature 377, 83–86.
Harrist LM, Bogan DJ, Mayer U, Kaufman SJ, Kornegay JN, Ervasti JM (2007). Cytoplasmic gamma-actin expression in diverse animal models of muscular dystrophy. Neurogenetics 7, 291–300.
Hanft LM, Bogan DJ, Mayer U, Kaufman SJ, Kornegay JN, Ervasti JM (2008). Effect of calpain and proteasome inhibition on Ca2+-dependent proteolysis and muscle histopathology in the mdx mouse. FASEB J 22, 4190–4200.

Harft LM, Rybakova IN, Patel JR, Rafael-Fortney JA, Ervasti JM (2006). Cytoplasmic gamma-actin contributes to a compensatory remodeling response in dystrophin-deficient muscle. Proc Natl Acad Sci USA 103, 5385–5390.

Jaeger MA, Sonnemann KJ, Fitzsimons DP, Prins KW, Ervasti JM (2009). Context-dependent functional substitution of alpha-skeletal actin by gamma-cytoplasmic actin. FASEB J 23, 2205–2214.

Knight PJ, Trinick JA (1982). Preparation of myofibrils. Methods Enzymol 85, B9–12.

Law DJ, Allen DL, Tidball JG (1994). Talin, vinculin and DRP (utrophin) concentrations are increased at mdx myotendinous junctions following onset of necrosis. J Cell Sci 107, 1477–1483.

Littlefield R, Almenar-Queralt A, Fowler VM (2001). Actin dynamics at pointed ends regulates thin filament length in striated muscle. Nat Cell Biol 3, 544–551.

Littlefield R, Fowler VM (2002). Measurement of thin filament lengths by distributed deconvolution analysis of fluorescence images. Biophys J 82, 2548–2564.

Littlefield RS, Fowler VM (2008). Thin filament length regulation in striated muscle sarcomeres: pointed-end dynamics go beyond a nebulin ruler. Semin Cell Dev Biol 19, 511–519.

Mardahl-Dumesnil M, Fowler VM (2001). Thin filaments elongate from their pointed ends during myofibril assembly in Drosophila indirect flight muscle. J Cell Biol 155, 1043–1053.

McCarter GC, Steinhardt RA (2000). Increased activity of calcium leak channels caused by proteolysis near sarcolemmal ruptures. J Membr Biol 176, 169–174.

Mciak JJ, Warsing LC, Tibbs ME, Jasper JR, Jampel SB, Malik FI, Tankersley C, Wagner KR (2013). Fast skeletal muscle troponin activator in the dy2J muscular dystrophy model. Muscle Nerve 48, 279–285.

Miyauchi-Nomura S, Obinata T, Sato N (2012). Cofilin is required for organization of sarcomeric actin filaments in chicken skeletal muscle cells. Cytoskeleton (Hoboken) 69, 290–302.

Mourkioti F et al. (2013). Role of telomere dysfunction in cardiac failure in Duchenne muscular dystrophy. Nat Cell Biol 15, 895–904.

Mudry RE, Perry CN, Richards M, Fowler VM, Gregory CC (2003). The role of cofilin action. J Cell Sci 108 (Pt 2), 581–593.

Ochala J, Gokhin DS, Iwamoto H, Fowler VM (2014). Pointed-end capping by tropomodulin modulates actomyosin crossbridge formation in skeletal muscle fibers. FASEB J 28, 408–415.

Ono S, Ono K (2002). Tropomyosin inhibits ADF/cofilin-dependent actin filament dynamics. J Cell Biol 156, 1065–1076.

Ottenheijm CA, Hooijman P, DeChene ET, Stienen GJ, Beggs AH, Granzier H (2010). Altered myofilament function depresses force generation in patients with nebulin-based nemaline myopathy (NEM2). J Struct Biol 170, 334–343.

Ottenheijm CA, Witt CC, Stienen GJ, Labet S, Beggs AH, Granzier H (2009). Thin filament length dysregulation contributes to muscle weakness in nemaline myopathy patients with nebulin deficiency. Hum Mol Genet 18, 2359–2369.

Perrin BJ, Amann KJ, Huttenlocher A (2006). Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration. Mol Biol Cell 17, 239–250.

Rahimov F, Kunkel LM (2013). The cell biology of disease: cellular and molecular mechanisms underlying muscular dystrophy. J Cell Biol 201, 499–510.

Rybakova IN, Humston JL, Sonnemann KJ, Ervasti JM (2006). Dystrophin and utrophin bind actin through distinct modes of contact. J Biol Chem 281, 9996–10001.

Rybakova IN, Patel JR, Davies KE, Yurchenco PD, Ervasti JM (2002). Utrophin binds laterally along actin filaments and can couple costameric actin with sarcolemma when overexpressed in dystrophin-deficient muscle. Mol Biol Cell 13, 1512–1521.

Sacco A et al. (2010). Short telomeres and stem cell exhaustion model Duchenne muscular dystrophy in mdx/mTR mice. Cell 143, 1059–1071.

Selsby J, Morris C, Morris L, Sweeney L (2012). A proteasome inhibitor fails to attenuate dystrophic pathology in mdx mice. PLoS Curr 4, e4f84d94dc8930.

Selsby J, Pendrak K, Zadel M, Tian Z, Pham J, Carver T, Acosta P, Barton E, Sweeney HL (2010). Leupeptin-based inhibitors do not improve the mdx phenotype. Am J Physiol Regul Integr Comp Physiol 299, R1192–1201.

Shimizu N, Obinata T (1986). Actin concentration and monomer-polymer ratio in developing chicken skeletal muscle. J Biochem 99, 751–759.

Spencer MJ, Croall DE, Tidball JG (1995). Calpains are activated in necrotic fibers from mdx dystrophic mice. J Biol Chem 270, 10909–10914.

Spencer MJ, Tidball JG (1992). Calpain concentration is elevated although net calcium-dependent proteolysis is suppressed in dystrophin-deficient muscle. Exp Cell Res 203, 107–114.

Sussman MA, Baque S, Ulhnr CS, Daniels MP, Price RL, Simpson D, Terracio L, Keders L (1998). Altered expression of tropomodulin in cardiomyocytes disrupts the sarcomeric structure of myofibrils. Circ Res 82, 94–105.

Tidball JG, Spencer MJ (2000). Calpains and muscular dystrophies. Int J Biochem Cell Biol 32, 1–5.

Turner FR, Schultz R, Ganguly B, Steinhardt RA (1993). Proteolysis results in altered leak channel kinetics and elevated free calcium in mdx muscle. J Membr Biol 133, 243–251.

Wadowsky KM, Li L, Rodriguez JE, Min JN, Bogan D, Gonzalez J, Patterson C, Kornejag NY, Willis M (2011). Regulation of the calpain and ubiquitin-proteasome systems in a canine model of muscular dystrophy. Muscle Nerve 44, 553–562.

Weber A, Pennise CR, Babcock GG, Fowler VM (1994). Tropomodulin caps the pointed ends of actin filaments. J Cell Biol 127, 1627–1635.

Weber A, Pennise CR, Fowler VM (1999). Tropomodulin increases the critical concentration of barbed end-capped actin filaments by converting ADP(Pi)-actin to ADP-actin at all pointed filament ends. J Biol Chem 274, 34637–34645.

Wenckel-Drake JD, Okita JR, Aniss DS, Kunicki TJ (1991). Activation of calpain I and hydrolysis of calpain substrates (actin-binding protein, glycoprotein Ib, and talin) are not a function of thrombin-induced platelet aggregation. Arterioscler Thromb 11, 882–891.

Yamashiro S, Gokhin DS, Kimura S, Nowak RB, Fowler VM (2012). Tropomodulin: pointed-end capping proteins that regulate actin filament architecture in diverse cell types. Cytoskeleton (Hoboken) 69, 337–370.

Yamashiro S, Speicher KD, Speicher DW, Fowler VM (2010). Mammalian tropomodulins nucleate actin polymerization via their actin monomer binding and filament pointed-end capping activities. J Biol Chem 285, 33265–33280.