Sorting of a nonmuscle tropomyosin to a novel cytoskeletal compartment in skeletal muscle results in muscular dystrophy

Anthony J. Kee,[1] Galina Schevzov,[2,5] Visalini Nair-Shalliker,[1] C. Stephen Robinson,[1] Bernadette Vrhovski,[2] Majid Ghoddusi,[1] Min Ru Qiu,[3] Jim J.-C. Lin,[4] Ron Weinberger,[2,5] Peter W. Gunning,[2] and Edna C. Hardeman[1]

[1]Muscle Development Unit, Children's Medical Research Institute, Wentworthville, New South Wales 2145, Australia
[2]Oncology Research Unit and [3]John Douglass Centre for Structural Pathology, The Children's Hospital at Westmead, Parramatta, New South Wales 2145, Australia
[4]Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242
[5]Discipline of Pediatrics and Child Health, University of Sydney, Sydney, New South Wales 4000, Australia

Tropomyosin (Tm) is a key component of the actin cyto-skeleton and >40 isoforms have been described in mammals. In addition to the isoforms in the sarcomere, we now report the existence of two nonsarcomeric (NS) isoforms in skeletal muscle. These isoforms are excluded from the thin filament of the sarcomere and are localized to a novel Z-line adjacent structure. Immunostained cross sections indicate that one Tm defines a Z-line adjacent structure common to all myofibers, whereas the second Tm defines a spatially distinct structure unique to muscles that undergo chronic or repetitive contractions. When a Tm (Tm3) that is normally absent from muscle was expressed in mice it became associated with the Z-line adjacent structure. These mice display a muscular dystrophy and ragged-red fiber phenotype, suggestive of disruption of the membrane-associated cytoskeletal network. Our findings raise the possibility that mutations in these tropomyosin and these structures may underpin these types of myopathies.

Introduction

The actin filament system is involved in a large array of functions that touch almost all aspects of the life of a cell. These vary from cell movement and cytokinesis to cell signaling and intracellular trafficking. Therefore, it has become an important challenge to understand how one filament system can independently regulate such a diverse range of cellular processes.

The actin filament is a double-stranded helical polymer of actin, the majority of which contain a tropomyosin polymer running along the helical groove (Flicker et al., 1982). Whereas actin is a globular protein that exists as monomer and polymer, tropomyosin is a rodlike head-to-tail dimer that is only known to exist in association with actin (Flicker et al., 1982; Matsumura et al., 1983). Both components, actin and

tropomyosin, have been shown to contribute to the physical properties of the microfilament (Kojima et al., 1994).

Actin and tropomyosin are encoded by multigene families and tropomyosin is also subject to extensive alternative splicing (Herman, 1993; Pittenger et al., 1994). Genetic manipulation has demonstrated that these isoforms are not redundant but rather encode different functional information. Cardiac α-actin is essential for normal cardiac function (Kumar et al., 1997), β-actin is required for cell spreading and motility (Schevzov et al., 1992; Kislauskis et al., 1997), and α-smooth muscle actin controls the contractility of myofibroblasts (Ronnov-Jessen and Petersen, 1996). Elevated expression of γ-actin disrupts stress-like organization in myoblasts (Schevzov et al., 1992) and sarcomere organization in cardiomyoctes (von Arx et al., 1995). Similarly, the Tm5NM-1 tropomyosin is required for melanoma cell motility (Miyado et al., 1996), α-fast tropomyosin is required for normal cardiac function (Thierfelder et al., 1994; Bottinelli et al., 1998; Muthu-

Abbreviations used in this paper: EDL, extensor digitorum longus; EOM, extraocular muscles; H&E, hematoxylin and eosin; MHC, myosin heavy chain; NS, nonsarcomeric; Tm, tropomyosin.

chamy et al., 1999), and the Tm1 and Tm2 tropomyosins are required to restore normal microfilament organization to cancer cells (Prasad et al., 1993; Boyd et al., 1995; Gimona et al., 1996). A specific tropomyosin isoform is required for correct mRNA targeting in *Drosophila* (Erdelyi et al., 1995) and the γ-TM gene is essential for embryonic development and embryonic stem cell viability (Hook et al., 2004).

The tropomyosins also show a variety of isoform-specific protein properties. The strength of binding to actin differs between tropomyosin isoforms although the original observation of tighter binding of the larger sized tropomyosins (Matsumura and Yamashiro-Matsumura, 1985) does not hold for some specific smaller tropomyosins (Pittenger et al., 1995). Tropomyosin isoforms also differentially protect actin filaments from severing by gelsolin (Ishikawa et al., 1989a,b) and regulate both myosin motor mechanochemistry (Fanning et al., 1994) and the sorting of myosin motors (Bryce et al., 2003). The azimuthal position assumed by tropomyosin on an actin filament also differs between isoforms and is additionally influenced by the actin isoform (Lehman et al., 2000). Therefore, it is clear that the properties of actin filaments are likely to differ depending on both the actin and tropomyosin isoform composition of the filament.

The extensive sorting of tropomyosin and actin isoforms to different intracellular locations provides two significant advantages to the cell (Gunning et al., 1998a,b). On the one hand, it allows the cell to independently control the supply of microfilament building blocks to different cellular sites. On the other hand, it provides a mechanism to regulate the functional properties of microfilaments at these sites (Weinberger et al., 1996; Schevzov et al., 1997; Hannan et al., 1998; Percival et al., 2000). The majority of these observations concerning tropomyosins have been made in neurons both in vivo and in vitro (Gunning et al., 1998b), in fibroblasts (Lin et al., 1988), synchronized NIH3T3 cells (Percival et al., 2000, 2004), epithelial cells (Temm-Grove et al., 1998; Dalby-Payne et al., 2003), and Golgi-derived vesicle fractions from rat liver (Heimann et al., 1999). Actin isoform sorting has also been observed in skeletal muscle (Prasad et al., 1993; Rybakova et al., 2000), smooth muscle (North et al., 1994), and neurons (Weinberger et al., 1996). Thus, the combination of isoform sorting and functional differences between isoforms provides a potentially powerful mechanism to segregate and independently regulate the myriad functions of actin filaments.

Isoform sorting of actins in skeletal muscle suggests the existence of a number of separate actin filament systems. One system provides the thin filament component of the sarcomere, which interdigitates with the myosin containing thick filaments. The thin filaments, also known as sarcomeric actin filaments, are composed of specific striated muscle α-actins and tropomyosins. A second filament system has been detected with a γ-actin antibody. Staining for γ-actin reveals its presence associated with costameres (Craig and Pardo, 1983; Rybakova et al., 2000), mitochondria (Pardo et al., 1983), and the Z-line (Nakata et al., 2001). This suggests the possibility of a γ-actin containing filament system that connects the myofibrils to the costameres.

We demonstrated previously that muscle differentiation is accompanied by down-regulation of nonsarcomeric (NS)
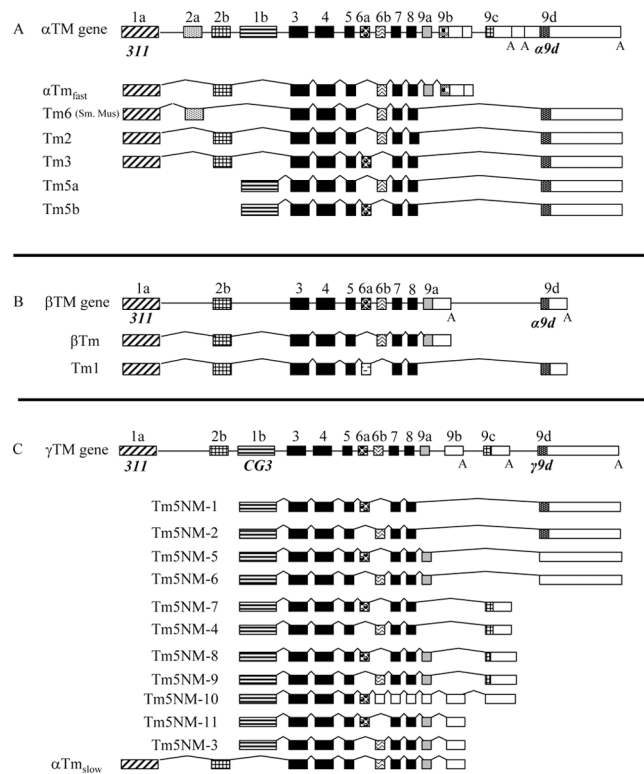

Figure 1. **Exon composition of tropomyosin (Tm) isoforms.** The α-TM (A), β-TM (B), and γ-TM (C) gene organization is shown, with boxes representing exons, lines introns, and unshaded parts of the exons untranslated regions. Homologous exons of the three genes are shown in the same shading pattern. Constitutive exons that are present in all known isoforms are represented by solid black boxes. A vertical bar and the letter A represent alternate polyadenylation sites. Antibodies used in this study with the specific exons they detect are shown in italics on the gene maps. All possible isoforms from the three genes are not depicted. The *311* antibody detects all isoforms containing exon 1a which includes sarcomeric (αTm_{fast}, βTm, and αTm_{slow}) and NS Tms, and the α*9d* antibody detects 9d containing isoforms from the α-TM and β-TM genes. The γ-TM gene-specific antibodies *CG3* and γ*9d* recognize isoforms containing exons 1b and 9d, respectively.

tropomyosins and induction of muscle isoforms (Gunning et al., 1990). It was noted, however, that some specific nonmuscle tropomyosins persist in adult muscle. We have used our repertoire of tropomyosin antibodies to characterize these isoforms in different adult skeletal muscles of the mouse. Two spatially distinct populations of tropomyosin-associated microfilaments are described: one in the sarcomeric compartment and the other at the myofiber periphery. Two tropomyosin isoforms, one (Tm5NM1) found in all muscles and a second novel product from the γ-gene (Tm5NM-34kd) found in a specific subset of muscles, localized adjacent to the Z-line in longitudinal sections. Further characterization of Tm localization in cross sections revealed that these two tropomyosins reside in separate filament systems. Tm5NM1 is particularly concentrated at the myofiber periphery and at lower amounts within the myofiber, presumably located between myofibrils. An anti–γ-actin antibody colocalized with this nonmuscle Tm at the periphery and within the fiber indicating that a γ-actin forms the backbone of this filament system. The novel Tm isoform
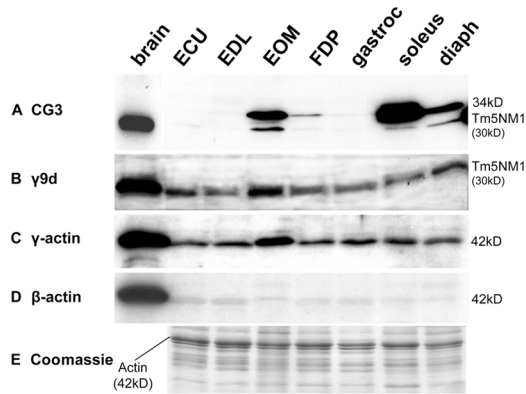
Figure 2. **Expression of NS γ-TM products and nonmuscle actins in skeletal muscles.** Shown are representative Western blots of protein (10 μg) from a range of mouse skeletal muscles and brain using antibodies that detect NS Tms from the γ-TM gene (A and B), γ-actins (C), and β-actin (D). The *CG3* antibody (A) specifically recognizes the γ-TM 1b exon and detects all known NS products from this gene, whereas the γ*9d* antibody (B) recognizes a subset of these NS Tms, those that contain exon 9d (Fig. 1 C). A novel Tm isoform was detected at high levels in a subset of the muscles examined (EOM, soleus, and diaphragm), a 34-kD protein from γ-TM containing exon 1b (A, *CG3* antibody). This novel isoform has not been detected in nonmuscle tissues. Both γ- and β-actin were detected in all muscles (C and D, respectively), but the levels were far greater for the former versus later isoforms. (Note that exposure to film was 45 s for γ-actin vs. 20 min for β-actin.) The γ-actin antibody also detects γ-smooth actin and so some of the signal with this antibody may be this isoform. (E) A representative Coomassie-stained gel used for the Westerns showing equal protein loading for the muscle extracts. Muscle abbreviations: ECU, extensor carpi ulnaris; EDL, extensor digitorum longus; EOM, extraocular muscles; FDP, flexor digitorum profundus; gastroc, gastrocnemius; quad, quadriceps; diaph, diaphragm.

Figure 3. **Tm5NM-34kd is expressed in slow fibers, whereas the γ*9d* isoforms are expressed in all major fiber types.** Consecutive transverse sections (7 μm) through the soleus muscle shows that fibers that were positive for the *CG3* antibody (A and B, asterisks) were slow fibers (stained with the slow myosin heavy chain (MHC) antibody in B. Sections were stained with the primary antibodies followed by peroxidase-conjugated goat/anti–mouse secondary antibodies. In contrast to *CG3*, γ*9d* stained all fibers in soleus (C) and EDL (E) muscles. Note that between them the soleus and EDL contain all adult skeletal muscle fiber types (types I, IIA, IIX, and IIB). Negative (−ve) control sections (D and F) with the secondary antibody (donkey anti–sheep Alexa 488) confirms the specificity of the staining with γ*9d*. Bars, 40 μm.

(Tm5NM-34kd) is located exclusively within the myofibers and in a separate filament system which does not colocalize with γ-actin. We have incorporated an inappropriate Tm into the Z-line adjacent structure by overexpressing a high molecular weight NS Tm, Tm3, that is not normally present in muscle. This results in phenotypes in these mice that are characteristic of muscular dystrophy and ragged-red fibers (subsarcolemmal accumulation of mitochondria).

Results

NS Tms and NS actin are expressed in skeletal muscle

The large diversity of tropomyosin isoforms results from multiple promoter initiation sites and extensive alternate splicing from four genes (isoforms from three genes are shown in Fig. 1). We and others have developed antibodies that detect a large variety of NS tropomyosins from the TM genes (Fig. 1). These antibodies were raised against peptides encoded by specific exons, most of which recognize gene-specific products (e.g., γ*9d*). Using these antibodies, it has been established in nonmuscle cells that the repertoire of Tms expressed is both cell-type specific, and spatially segregated within a cell (Lin et al., 1997; Gunning et al., 1998a,b). We have previously observed that after muscle differentiation, at least one mRNA encoding a cytoskeletal Tm from the γ-TM gene persists in adult muscle. Antibodies directed against the amino- (*CG3*) and carboxy-terminal (γ*9d*) exons

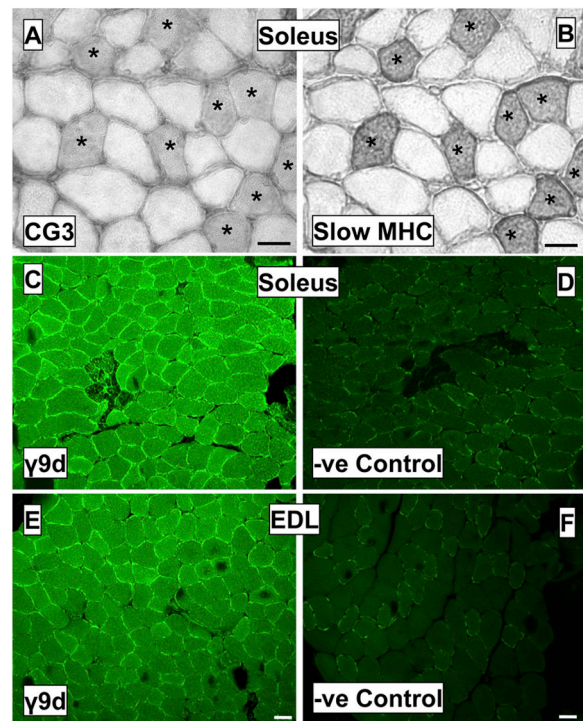of the γ-TM gene were used to define protein expression and location in adult muscle (Fig. 1).

Western blots reveal that adult skeletal muscles express two NS Tms at significant levels (Fig. 2, A and B). The expression of NS Tms varies widely between different muscles, with the extraocular muscle (EOM), soleus, and diaphragm muscles having particularly high levels of NS Tms. A novel 34-kD muscle-specific isoform was detected by the *CG3* antibody in these muscles (very low amounts in flexor digitorum profundus muscle also; Fig. 2 A). All known products recognized by *CG3* migrate at 30 kD (e.g., Tm5NM1), therefore this is likely to be a larger protein with a novel carboxy terminus because it is not recognized by the γ*9d* antibody (Fig. 2 B; no 34-kD band in the Western blots) or antibodies against the other carboxy-terminal exons from this gene (not depicted). In contrast to the muscle-specific expression of this novel isoform, γ*9d* detected a band that was present in all skeletal muscles examined (Fig. 2 B). This band (30 kD) is likely to be Tm5NM1, as a band of this size was not detected with an antibody (WS5/9d; Percival et al., 2004) that preferentially recognizes Tm5NM2, the only other exon 9d-containing product from the γ-TM gene (Dufour et al.,
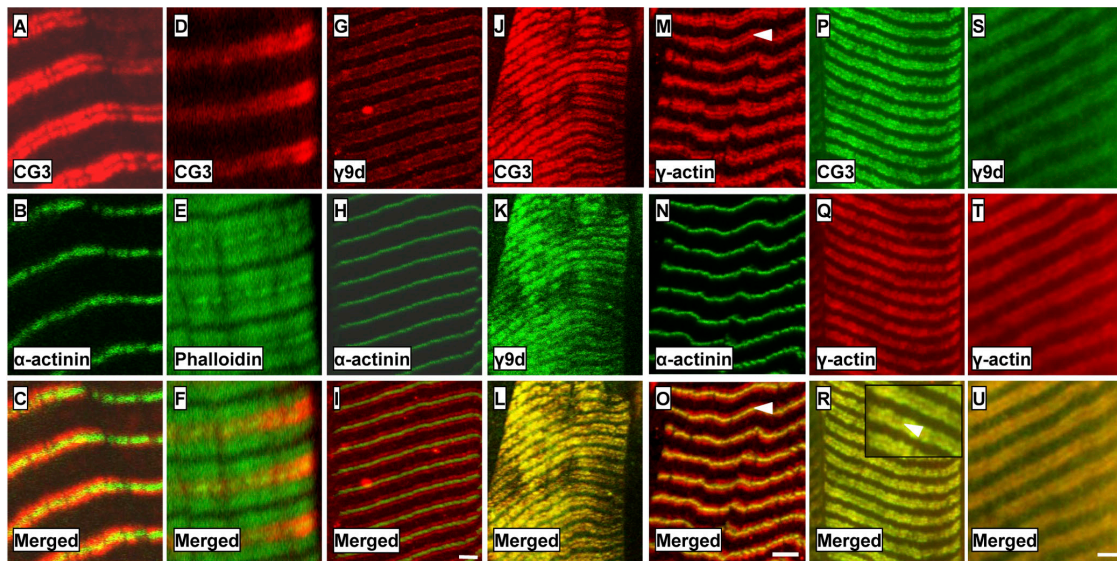
Figure 4. **The NS Tms from the γ-gene and a γ-actin are colocalized to a novel Z-line adjacent region of the sarcomere.** Confocal immuno-fluorescent images of semi-thin (0.5–1.0 μm) longitudinal sections through adult soleus muscle show that Tms detected by *CG3* and γ*9d* (A, C, D, F, G, and I, red signal) are localized to a restricted area either side of Z-line (B, C, H, and I, delineated by the green α-actinin staining), but not including the Z-line. The restricted localization of these NS Tms to either side of the Z-line is in marked contrast to the broad region of the actin thin filament of the sarcomere (E and F, phalloidin). In longitudinal sections there appears to be little difference in the Z-line adjacent localization of the Tms detected by *CG3* and γ*9d* (J–L). Antibodies to a γ-actin also stained the Z-line adjacent region (M–O) and this staining was coincident with the staining for both *CG3* (P–R) and γ*9d* (S–U). In some sections, γ-actin staining was observed at the Z-line (M and O, arrowheads; R, inset). Double staining of muscle sections was performed by applying both the primary/secondary antibody pairs sequentially. Bars, 2.5 μm.

1998a). We have also examined the expression of nonmuscle actins that are known to be present in skeletal muscle, γ-actin, and β-actin (Fig. 2, C and D, respectively). γ-Actin and β-actin were expressed in all muscles examined, but the level of expression of γ-actin was much greater than β-actin (Fig. 2, C and D, respectively). Because the γ-actin antibody also detects γ-smooth actin (not depicted) we cannot exclude the possibility that at least some of the γ-actin signal is due to this isoform.

The novel CG3 isoform (Tm5NM-34kd) was expressed at particularly high levels in the slow-twitch soleus muscle and in other specialized muscles (Fig. 2 A, EOM and diaphragm). To examine fiber-type specific expression of this isoform we stained serial transverse sections of soleus muscles with *CG3* and a slow myosin heavy chain (MHC) antibody. Only slow fibers were strongly positive for *CG3* (Fig. 3, A and B) suggesting that Tm5NM-34kd is expressed predominately in slow fibers in the soleus. High levels of this novel Tm were also expressed in the diaphragm and eye muscles (EOM), muscles that only have few (5–10% of total) slow fibers in rodents (Wieczorek et al., 1985; Seward et al., 2001) suggesting that Tm5NM-34kd may also be expressed in fast fibers of the diaphragm and the specialized fibers of the eye muscles (e.g., extraocular fast and slow tonic in the EOM). In contrast, the γ*9d* antibody, which detects Tm5NM1 (Fig. 2 B), stained all fibers of the soleus and extensor digitorum longus (EDL) muscles (Fig. 3, C and E, respectively), indicating that Tm5NM1 is in all adult skeletal muscle fibers (types I, IIA, IIX, and IIB) in the mouse. This data is consistent with the Western blots showing similar expression of Tm5NM1 in all muscles (Fig. 2 B).

NS Tms define a novel Z-line adjacent compartment in skeletal muscle

Studies in a number of nonmuscle systems have shown that the NS Tms are sorted to very specific intracellular locations (Gunning et al., 1998a,b). Therefore, we have stained longitudinal sections of muscle with the γ9d and CG3 antibodies to define the localization of Tm5NM1 and Tm5NM-34kd in muscle. Both antibodies produced strong striated staining that was distinct from the thin filament of the sarcomere. Specifically, both *CG3* and γ*9d* (Fig. 5, A, D, and G, red staining) showed thin lines of staining adjacent to the Z-line (Fig. 5, B and H, green α-actinin staining) that did not correspond to the entire actin thin filament (Fig. 5 F, compare red *CG3* and green phalloidin/filamentous-actin staining). The *CG3* and γ*9d* staining is likely to represent the localization of Tm5NM-34kd and Tm5NM1, respectively, as on Western blots these were the predominant Tms detected by these antibodies in the soleus muscle (Fig. 2, A and B, respectively). On longitudinal sections the two Tms colocalized with each other in this Z-line adjacent region (Fig. 4 L, yellow staining in the merged image) and both colocalized in this region with the γ-actin antibody (Fig. 4, P–U). Costaining of sections with antibodies to γ-actin and the Z-line protein α-actinin indicates that γ-actin is at the Z-line adjacent region (Fig. 4 O) and also at the Z-line itself (indicated by the colocalized yellow signal of γ-actin and α-actinin in Fig. 4 O). The NS Tm banding pattern around the Z-line does not correspond to any known pattern of Tm staining in skeletal myofibers. Together, the data suggests that a γ-actin and these NS Tms are part of a novel cytoskeleton adjacent to the Z-line.
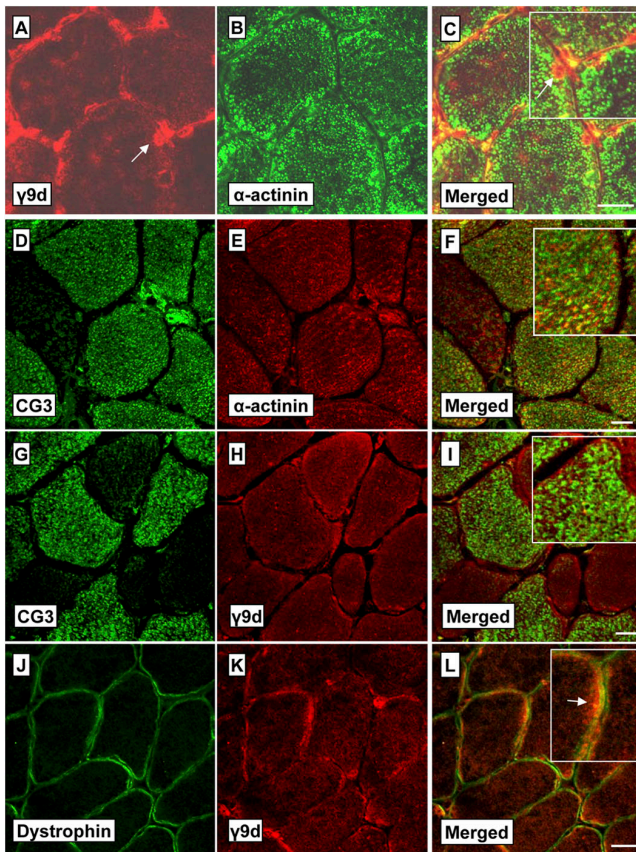
Figure 5. **NS Tms recognized by the CG3 and γ9d antibodies have distinct myofiber localization.** Shown are confocal images of transverse sections (7 μm) through soleus muscles stained with γ9d (A) or CG3 (D) and costained with α-actinin (B and E). The enlarged merged images (C and F, insets) indicate that the Tms recognized by γ9d and CG3 are not colocalized with α-actinin (labels myofibrils) and therefore are located outside the myofibrils. Further transverse sections (G–I) show that CG3 and γ9d stain separate regions within the myofiber (particularly notable in the enlarged inset, I). Cross sections costained with the membrane protein dystrophin (J) and γ9d (K) also show strong staining of γ9d at the myofiber periphery beneath the membrane (L, arrow in the enlarged inset). Bars, 20 μm.

Figure 6. **In transverse section, the γ9d Tms localize with a γ-actin, whereas the CG3 Tms do not.** Shown are confocal images of transverse sections (7 μm) through soleus muscles stained with γ9d (A) or CG3 (D) and costained with a γ-actin antibody (B and E). The merged images show that the Tms recognized by γ9d colocalize with a γ-actin within the myofiber and at the fiber periphery (C), whereas the CG3 Tms are not localized with γ-actin (F). In addition, signal for a γ-actin did not coincide with α-actinin (I, enlarged inset) indicating that γ-actin microfilaments are not closely associated with the myofibrils. The most intense γ-actin staining was at the myofiber periphery (B, E, H, and K), but the staining was mostly distinct from the sarcolemmal staining of dystrophin (L, inset). Bars, 20 μm.

The immunofluorescent microscopy on longitudinal sections indicated that the NS Tms recognized by CG3 and γ9d antibodies are present in a structure that exists adjacent to the Z-line. To gain more information about this structure we stained transverse sections of soleus muscle with these two antibodies and costained with the Z-line marker, α-actinin (Fig. 5, A–F). From the merged images it is evident that the pattern of staining of γ9d and CG3 is separate from α-actinin (Fig. 5, C and F, respectively); the green and red signals do not overlap for the most part. As α-actinin stains the Z-line within individual myofibrils these images suggest that Tm5NM1 and the novel 34-kD Tm are likely to be part of microfilaments not closely associated with the myofibrils. Moreover, the Tms recognized by CG3 and γ9d appear to be part of separate filament systems as there was little colocalized signal with these antibodies on merged images (Fig. 5 I). Although γ9d (Tm5NM1) stained areas within the myofiber, the strongest staining for this antibody was at the myofiber periphery (Fig. 5, A and K). However,
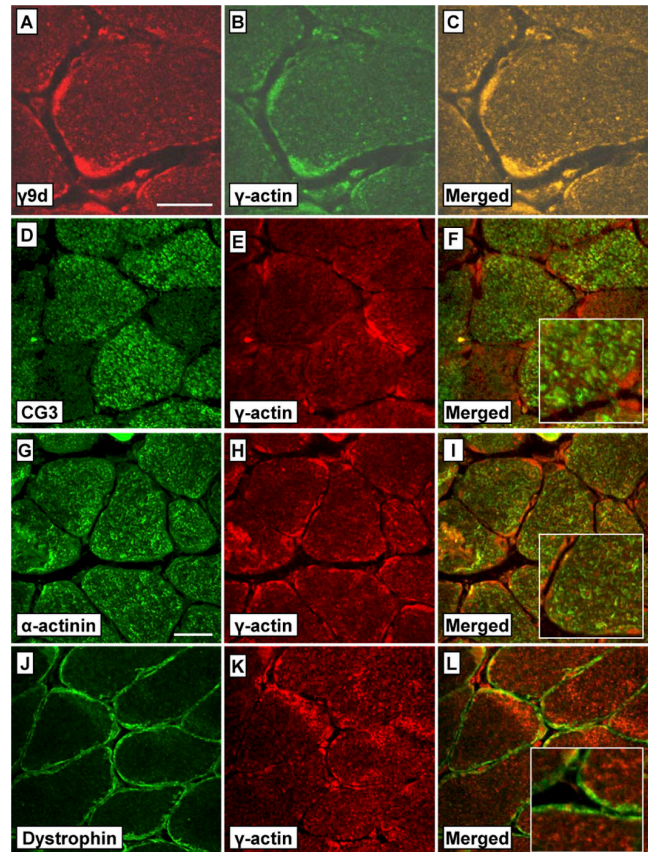
γ9d staining did not coincide with dystrophin, a classical membrane marker, but rather stained the subsarcolemmal space in a discontinuous fashion (Fig. 5 L, inset).

We next stained transverse sections of soleus muscle with the γ-actin antibody and the NS Tm antibodies to determine whether a γ-actin is part of the novel NS Tm filament structure in muscle (Fig. 6). From the merged images it is evident that there is almost complete colocalization of γ9d and γ-actin both within the myofiber and at the fiber periphery (Fig. 6 C). In contrast, the staining of CG3 appeared to be separate from γ-actin (Fig. 6 F, inset). This suggests that in skeletal muscle Tm5NM1 (detected by γ9d) is part of a γ-actin filament network, whereas the novel CG3 Tm (Tm5NM-34kd) associates with a different actin filament system. γ-Actin staining was not coincident with α-actinin (Fig. 6 I) and was concentrated in the subsarcolemmal space separate from dystrophin (Fig. 6 L). This pattern of staining is similar to γ9d and is consistent with a γ-actin–Tm5NM1 filament system located between the myofibrils and in the subsarcolemmal space.
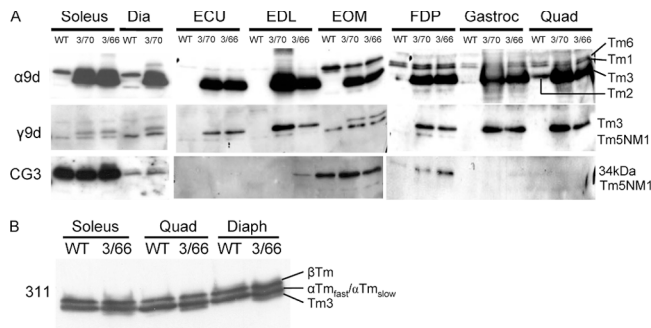
Figure 7. **Expression of high levels of Tm3 in muscle has little effect on expression of sarcomeric and NS Tms.** Western blots of 6-mo-old Tm3 skeletal muscle showing the influence of high levels of Tm3 expression on (A) NS or (B) sarcomeric Tm isoforms. Results for both wild-type (WT) and high-expressing transgenic lines (3/70 and 3/66) are shown. NS Tms containing exon 9d from the α- and β-TM genes are recognized by α*9d*. Products containing exon 1b and exon 9d from the γ-TM gene are recognized by *CG3* and γ*9d*, respectively. A Tm3 band is detected by the γ*9d* antibody in Tm3 mice (3/70 and 3/66) due to cross-reactivity of the antibody with exon 9d products from the α-TM gene. The *311* antibody recognizes Tms containing exon 1a (Fig. 1), which includes Tm3 and the three sarcomeric Tms (αTm$_{fast}$, αTm$_{slow}$, and βTm). Under the electrophoretic conditions used the αTm$_{fast}$ and αTm$_{slow}$ isoforms are seen as a single band. See Fig. 2 legend for muscle abbreviations.

An ectopic NS Tm, Tm3, incorporates into the Z-line adjacent structure

As the NS Tms appear to be part of different cytoskeletal structures in muscle we were interested to examine what effect the expression of an inappropriate cytoskeletal Tm would have on the Tms defining these structures. Therefore, we have studied transgenic mice that express an NS Tm, Tm3, that is not normally expressed in skeletal muscle (Fig. 7 A). Western blots with the α9d antibody, show that transgenic lines 3/66 and 3/70 accumulate very high levels of Tm3 in a wide range of muscles (Fig. 7 A). Tm3 is also detected by γ*9d* in the transgenic mice (Fig. 7 A), as there is some cross-reactivity of the antibody to exon 9d from the α- and β-TM genes.

The levels of NS Tm3 in the transgenic mice do not, however, exceed that of the muscle sarcomeric Tms (Fig. 7 B). A Western blot of quadriceps muscle from control WT and line 3/66 mice probed with an antibody (311) to exon 1a of the α-, β-, and γ-TM genes is shown in Fig. 7 B. Strong bands corresponding to the sarcomeric Tms (αTm$_{slow}$, αTm$_{fast}$, and βTm) are evident in both control and 3/66 muscles. In addition, a band corresponding to Tm3 is seen migrating slightly faster than α-Tm. Thus, the levels of Tm3 in these mice approach that of the sarcomeric Tms and there is no indication of a compensating down-regulation of the sarcomeric isoforms.

Examination of Western blots probed with the α*9d*, γ*9d*, and *CG3* antibodies (Fig. 7 A) shows that expression of Tm3 in skeletal muscle has little effect on the expression of many other NS Tms (e.g., Tm1, Tm2, and Tm6). The only exception appears to be increased expression of the novel Tm5NM-34kd *CG3* isoform in the flexor digitorum profundus muscle (Fig. 7 A).
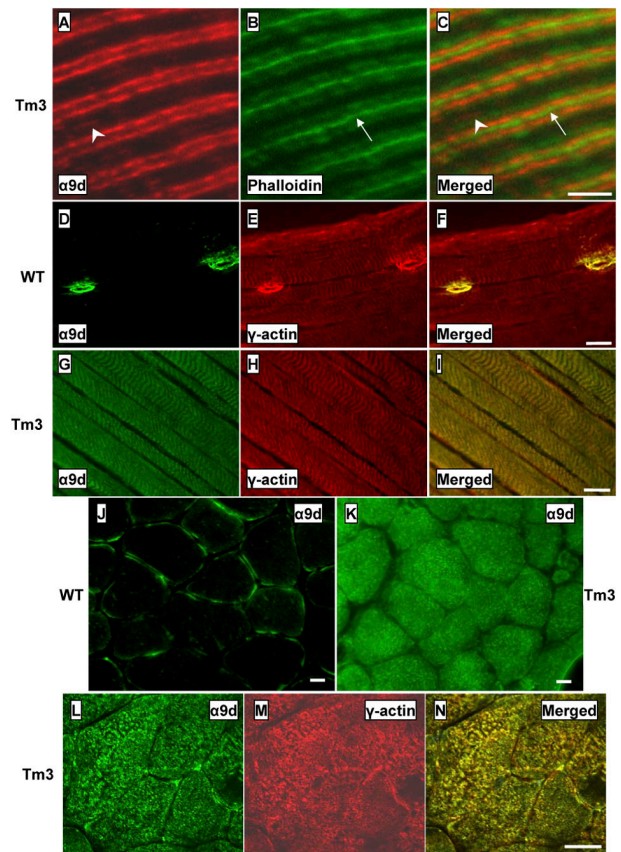

Figure 8. **Ectopically expressed Tm3 accumulates adjacent to the Z-line and at the M-line.** Shown are immunofluorescent confocal images of longitudinal and transverse sections of soleus muscle from adult Tm3 transgenic (line 3/66) and wild-type (WT) mice. WT soleus muscle contains two main Tm isoforms (Tm1 and Tm6; Fig. 7 A) recognized by the α*9d* antibody. Longitudinal and transverse sections (7 μm) of WT muscle indicate that these isoforms are present in structures outside of the myofiber and at the myofiber periphery (D and J, respectively). Tm3 (also detected by the α*9d* antibody; Fig. 1 A), is absent from WT muscle (Fig. 7 A), but in the Tm3 mice the ectopic protein is located at the Z-line adjacent region (A–C, 0.5–1.0 μm longitudinal sections; Z-line marked by arrows) and at the M-line (A and C, arrowheads). Tm3 localized mainly to the sarcomeric compartment (G and K) and was colocalized with the γ-actin antibody in longitudinal and cross sections (I and N, respectively). Bars: (A–C) 2.5 μm; (D–I) 40 μm; and (J–N) 20 μm.

The ectopic Tm3 protein localized to the same Z-line adjacent region as the endogenous γ-TM gene isoforms (Tm5NM1 and Tm5NM-34kd; Fig. 8). Very strong staining either side of the Z-line can be seen in soleus longitudinal sections with the α*9d* antibody (Fig. 8, A and C). Ectopic Tm3 was mainly restricted to the cytoplasmic region of the myofiber (Fig. 8, G, K, and L). This staining is specific for ectopic Tm3 as although this antibody detects a number of different endogenous isoforms in the soleus muscle (Fig. 7 A) none of these are present in the cytoplasmic sarcomeric regions of the muscle (Fig. 8, D and J). In longitudinal and cross sections, ectopic Tm3 colocalized with the γ-actin antibody (Fig. 8, I and N, respectively). This is consistent with an association of Tm3 with a Z-line adjacent γ-actin cytoskeleton.
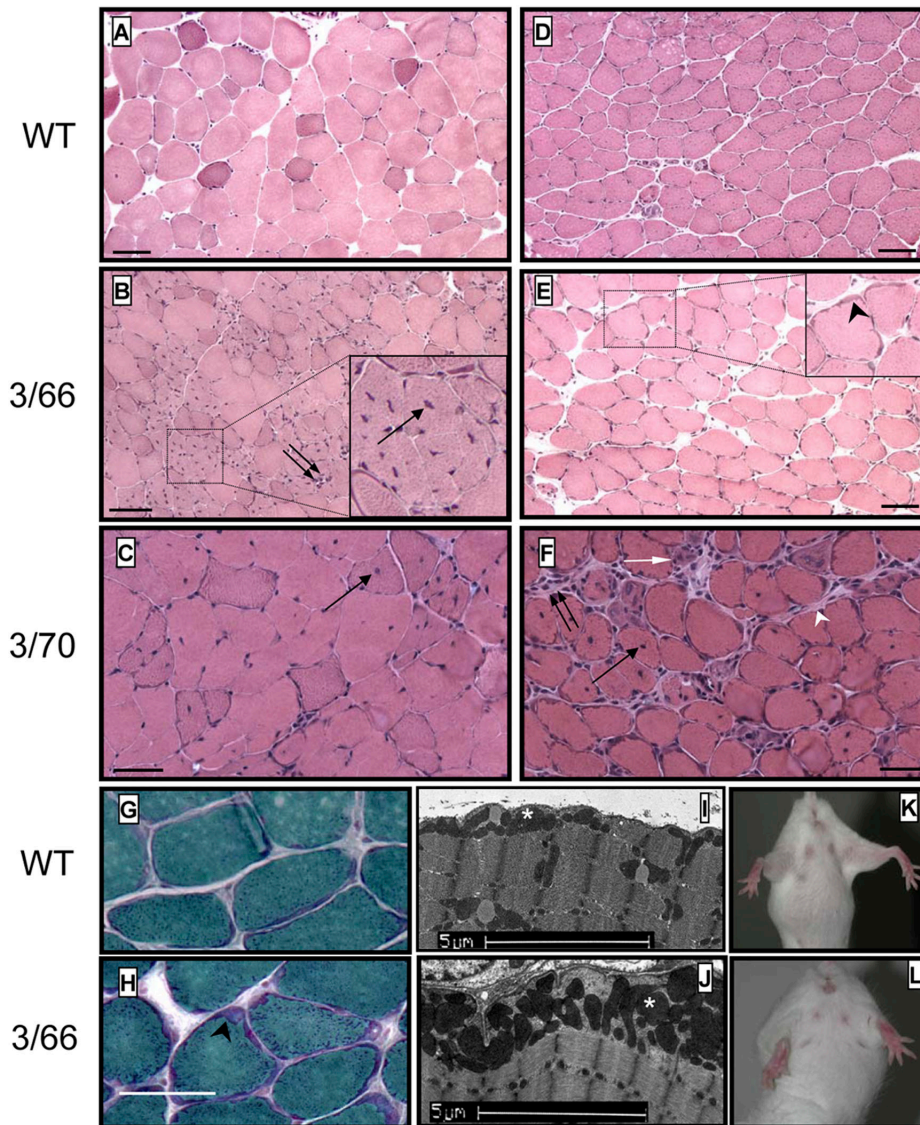
Figure 9. **Inappropriate expression of an NS Tm (Tm3) results in muscular dystrophy phenotype and ragged-red fibers.** The Tm3 mice have a number of features characteristic of muscular dystrophy in quadriceps (A–C) and soleus muscles (D–F). Features characteristic of muscular dystrophy shown in H&E stained transverse sections (A–F) include: areas of regenerating fibers with centralized nuclei (B, C, and F, black arrows), myofiber size variability (B, C, and F), and regions of fiber degeneration (F, white arrow), macrophage infiltration (B and F, black double arrows) and fibrosis (F, white arrowhead). Soleus muscle from Tm3 mice also has the characteristic accumulation of sarcolemmal mitochondria that is associated with ragged red fibers. Mitochondrial accumulations (black arrowheads) were observed in Gomori-Trichrome (G) and H&E stained (E) sections in of soleus of line 3/66 mice. The large increase in number and size of mitochondria beneath the sarcolemma is clearly evident in electron micrographs from Tm3 mice (J) compared with WT mice (I). Asterisks in I and J mark the subsarcolemmal mitochondria. Bars, A–H, 40 μm. When suspended by the tail the Tm3 mice (line 3/66; L), unlike the WT mice (K), are unable to extend their hindlimbs away from the body. This phenomenon has been observed in a mouse model of muscular dystrophy (Bittner et al., 1999).

Tm3 expression elicits a dystrophic and ragged-red fiber phenotype

We next examined the effect of expression of Tm3 on muscle pathology (Fig. 9). Muscles from Tm3 mice showed classical signs of muscular dystrophy as visualized by hematoxylin and eosin (H&E) staining of cross sections. This includes large areas of myofiber degeneration, macrophage infiltration and fibrosis and regions of small regenerating fibers with centralized nuclei (Fig. 9, B, C, and F). Dystrophic features were observed in both transgenic lines (3/66 and 3/70) and in a number of different mus-

cles: the quadriceps (Fig. 9, B and C), soleus (Fig. 9 F), gastrocnemius, and back muscles (results not depicted for latter two sets of muscles).

Further histological examination showed that the Tm3 mice also have ragged-red fibers (Fig. 9, E and H). Ragged-red fibers are not normally associated with dystrophies but are a common feature of mitochondrial myopathies (Banker and Engel, 1994). Dark submembranous staining was clearly evident in Gomori-Trichrome (Fig. 9 H) and H&E stained sections (Fig. 9 E) from Tm3 mice. On EM examination this was shown to be an abnormally large accumula-

tion of mitochondria beneath the sarcolemma in Tm3/66 compared with WT mice (Fig. 9, J vs. I, respectively). The mitochondria were large in size and number, but the internal structure of the mitochondria appeared to be normal, as did the morphology of the inter-myofibrillar mitochondria.

The dystrophic features were associated with muscle weakness that is shown by the inability of the Tm3 mice to extend their limbs when held by the tail (compare WT and Tm3/66 mice in Fig. 9, K and L, respectively). This type of muscle dysfunction is similar to that observed in SJL mice, a natural mouse model for limb girdle muscular dystrophy 2B (Bittner et al., 1999). The Tm3 mice also have altered gait when running on a treadmill; they run with their hindlimbs closer to the body and with an arched back. Further evidence of dystrophy in the Tm3 mice is the raised levels of serum creatine kinase (1300 ± 150 U/L [mean ± SEM] and 300 ± 50 U/L for Tm3 [$n = 5$] and WT [$n = 4$] mice, respectively; P = 0.023, ANOVA). Elevated plasma creatine kinase is a characteristic feature of muscular dystrophies and is thought to be an indicator of loss of sarcolemma integrity. Therefore, we conclude that incorporation of Tm3 into the novel Z-line–associated actin–Tm filament network leads to mice with features of muscular dystrophy and ragged-red fiber myopathy.

Discussion

NS tropomyosin isoforms are expressed in skeletal muscle

There is increasing evidence for the existence of a cytoskeleton spatially separated from the contractile apparatus in skeletal muscle (Clark et al., 2002; Ervasti, 2003). β-Actin is enriched in the cytoskeleton associated with the postsynaptic folds of the neuromuscular junction (Hall et al., 1981; Lubit, 1984). In contrast, γ-actin is found in microfilaments associated with dystrophin at the costameres (Rybakova et al., 2000; Ervasti, 2003). γ-Actin is also detected in a structure which coaligns with α-actinin (Nakata et al., 2001) and associates with subsarcolemmal mitochondria as well (Pardo et al., 1983).

Previously, we found that during muscle differentiation there is a switch in Tm expression from NS to sarcomeric isoforms (Gunning et al., 1990). However, expression of a number of NS isoforms, particularly from the γ- and δ-TM genes, persisted in adult muscle. The more detailed analysis shown here using isoform specific antibodies demonstrates that the level of NS Tm expression is highly muscle specific. In addition to the known cytoskeletal Tm (Tm5NM1) that was expressed in all muscles, skeletal muscle also expresses a novel 34-kD product recognized by *CG3* that was abundant in a very restricted set of muscles, the EOM, the diaphragm, and the soleus muscle. There is no known EST corresponding to the novel γ-TM 34-kD product, although we have previously reported a novel, slow muscle specific transcript from this gene (Dufour et al., 1998b). The carboxy terminus of this product has not been characterized but may correspond to this novel 34-kD product.

The novel Tm5NM-34-kD protein, a product of the γ-TM gene, is abundant in slow fibers, but appears not to be restricted to slow fibers because it is abundant in muscles that contain few slow fibers (EOM and diaphragm). This unusual pattern of expression suggests this isoform has some
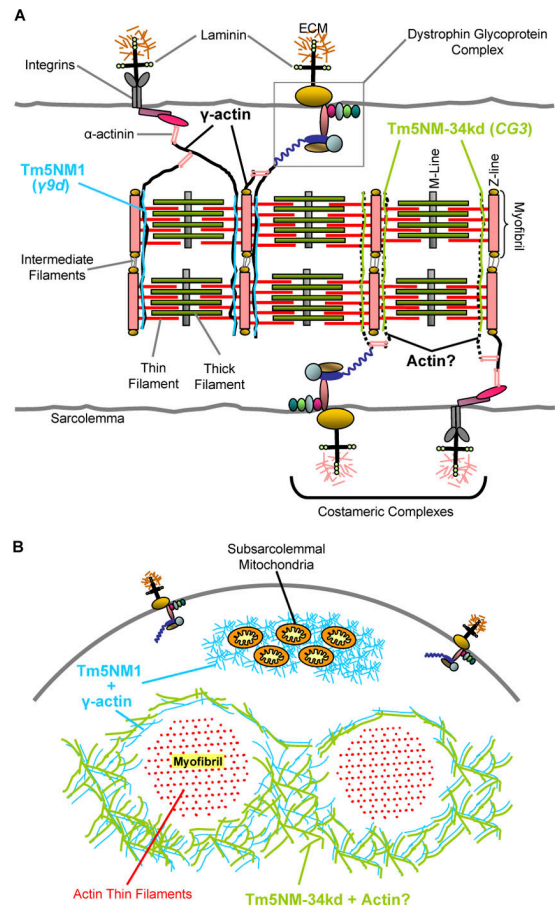

Figure 10. **Schematic representation of NS Tm localization in skeletal muscle.** Depicted are myofibers in longitudinal (A) and transverse section (B) showing the location of the NS Tms and γ-actin in relation to the major structures in skeletal muscle. (A) In the longitudinal representation, two main membrane/cytoskeletal junctions are depicted: (1) integrin-based focal adhesions and (2) the dystrophin glycoprotein complex. Hypothesized tropomyosin-associated γ-actin networks that link the sarcomeric arrays to the costamere are shown as black lines. Other filaments linking the M-line and Z-line to the membrane are not depicted. The results of this study suggest that the NS tropomyosins recognized by the γ9d and CG3 antibodies (Tm5NM1 and Tm5NM-34kd, respectively) are located adjacent to the Z-line in the sarcomeric space. Data suggest that Tm5NM1 associates with a γ-actin whereas the actin (broken black line) interacting with Tm5NM-34kd was not defined. Note that although α-actinin (pink) is only shown cross-linking γ-actin filaments, its main location is in the Z-line linking sarcomeric actin thin filaments from adjacent sarcomeres. (B) In the transverse representation, Tm5NM1 (blue) and Tm5NM-34kd (green) are shown in the intermyofibrillar space, the former in association with a γ-actin and the later with an unknown actin filament system. Also shown is Tm5NM1/γ-actin filaments at the subsarcolemmal region, a space also occupied by mitochondria. The data suggests that the Tm5NM1 filaments are in all adult fibers, whereas the Tm5NM-34kd structure is restricted to specialized subset of fibers. The ectopic Tm3 localizes to the Z-line–associated γ-actin cytoskeleton and results in muscular dystrophy and ragged-red fibers.

specialized function in specific myofibers in contrast to Tm5NM1, which is expressed in all muscle fibers. The muscles that contain high amounts of the novel Tm are characterized by chronic or frequent contractions. This suggests that Tm5NM-34kd may be associated with a structure unique to these fibers.

Tropomyosin isoforms define a novel Z-line–associated element in skeletal muscle

Using antibodies to γ-TM gene products (γ9d and CG3) and an antibody to a γ-actin, a novel structural compartment in muscle has been identified. This compartment is present at a very restricted area adjacent to the Z-line. Analysis of muscle transverse sections suggests that within this compartment there are at least two separate structures located in the intermyofibrillar space. One, labeled by γ9d, containing Tm5NM1, appears to be associated with a γ-actin filament network, and the other, defined by CG3 (Tm5NM-34kd), appears to be part of a separate filament system. The localization of Tm5NM1 and Tm5NM-34kd in the intermyofibrillar space aligned adjacent to the Z-line suggests there are filament networks that laterally interconnects the Z-line adjacent region of individual myofibrils (Fig. 10 A). This system would be analogous to the desmin intermediate filament network that laterally interlinks the Z-disks of each myofibril (Capetanaki, 2002).

A subsarcolemmal filament system containing Tm5NM1 (labeled by γ9d) and a γ-actin was also identified. These filaments were not evenly distributed around the periphery of the myofiber but were concentrated in discrete foci near the membrane and often extended some distance into the myofiber. Previous studies have shown association of γ-actin with the subsarcolemmal mitochondria (Pardo et al., 1983). Thus, the present findings are consistent with Tm5NM1 being part of this γ-actin cytoskeleton (Fig. 10 B).

There is increasing evidence that many signaling molecules are present in a region adjacent to the Z-line in skeletal muscle (Ervasti, 2003). Chisel, STARS, Arpp, myopalladin, enigma, FHL3, and myopodin locate to this region in skeletal muscle (Guy et al., 1999; Bang et al., 2001; Palmer et al., 2001; Arai et al., 2002; Tsukamoto et al., 2002; Coghill et al., 2003). The colocalization of these molecules suggests that there must be a scaffold that provides the structure to organize these molecules. This provides evidence for a structural network of signaling molecules that links the sarcomere to costameres and the ECM. The NS Tm–actin filament network described in the present investigation may be such a scaffold structure.

Incorporation of Tm3 into Z-LAC results in muscular dystrophy and ragged-red fibers

Transgenic mice that express Tm3 in their skeletal muscle display two significant pathologies. Because the Tm3 is specifically targeted to the Z-line–associated cytoskeleton, we propose that it is the inclusion of Tm3 into this structure which directly leads to the pathologies. The pathologies and localization were observed in two independent transgenic lines indicating that these pathologies are not due to transgene integration-site effects. The impact of Tm3 does not appear to be due to competing the endogenous Tms out of this structure because CG3 and γ9d staining is unchanged (unpublished data) and the levels of Tm5NM1 and Tm5NM-34kd are unchanged. This suggests that the γ-actin filaments that presumably provide the backbone for these microfilaments are not saturated with tropomyosin and can accommodate the ectopic expression of Tm3. This may also account for the specific

sorting of Tm3 to this site because these γ-actin filaments may be the major source of microfilaments lacking tropomyosin in skeletal muscle.

Mutations in a growing number of costameric proteins and proteins that interact with the costamere have been identified as causes of muscular dystrophies (Ervasti, 2003). The best-known example is dystrophin itself, which leads to dystrophies of differing severity depending on the specific mutations (Dalkilic and Kunkel, 2003). Recently, proteins that interact with the Z-line have also been shown to cause muscular dystrophies when mutated or absent (e.g., telethonin, calpain 3; Richard et al., 1995; Moreira et al., 2000). This suggests that it is a defect in the overall function of this complex that is responsible for the majority of dystrophies. The muscles of Tm3 transgenic mice display a phenotype characteristic of muscular dystrophy. Because Tm3 specifically localizes to the Z-line adjacent microfilaments, the data suggest that it is dysfunction of these filaments resulting from inappropriately targeted Tm3 that is leading to the dystrophic phenotype. Furthermore, this provides evidence that this microfilament network is an important structural element that directly or indirectly links the sarcomere to the sarcolemma, and that mutations in this structure may account for some currently unknown causes of muscular dystrophy.

The appearance of ragged-red fibers in the Tm3 transgenic lines suggests that the NS Tm cytoskeletal structures may influence mitochondrial function. It is notable that the desmin knockout mouse displays a similar mitochondria phenotype (Milner et al., 2000). Mitochondria are normally located beneath the sarcolemma and also near the Z-line between myofibrils. Previous studies have shown an association of γ-actin with subsarcolemmal mitochondria (Pardo et al., 1983; Nakata et al., 2001) and the present results are consistent with NS Tms (Tm5NM1) associated with these microfilaments. It is possible that the γ-actin Z-line–associated cytoskeleton and desmin play a role in anchoring mitochondria at this site. Disruption of this anchoring may leads to mitochondrial disruption on the one hand and migration to the cell periphery on the other.

In conclusion, the novel NS microfilament structure, together with desmin, may provide a physical link between the myofibrils, the costamere and the ECM. Alterations in the function of this novel structure may lead to muscular dystrophy and/or ragged-red fibers. The tropomyosin components of this Z-line–associated cytoskeleton should now be included as candidate genes in searches for mutations that cause these diseases.

Materials and methods

Generation of the Tropomyosin-3 (Tm3) transgenic mouse

The rat Tm3 cDNA under the control of the human β-actin promoter was removed from vector sequences with Kpn1 and EcoR1 (Roche Diagnostics). The amino acid sequence for human, rat, and mouse Tm3 are identical. Fertilized eggs were collected from superovulated FVB/NJ females, injected with the DNA, and transferred to pseudopregnant females according to Hogan et al. (1994). Transgenic mice were screened by Southern blot of DNA from mice tails, digested with SacI (Roche Diagnostics, Inc.), and probed with a 1-kb SacI fragment from the 5′ flanking region of the β-actin promoter. Wild-type mice were nontransgenic FVB/NJ littermates. The CMRI/CHW Animal Care and Ethics Committee approved this study.

Immunohistochemistry

Standard cryosectioning. Immediately after dissection, mouse muscles were coated in tissue freezing medium (Tissue-Tek O.C.T., ProSciTech), frozen in melting isopentane prechilled in liquid nitrogen and stored at −80°C. Muscles were sectioned in a cryostat microtome (HM500OM; Carl Zeiss MicroImaging, Inc.) at 7 μm, fixed in cold 2% PFA, and washed in PBS.

Semi-thin sectioning. Greater resolution of Z-line–associated structures was achieved with semi-thin (0.5–1.0 μm) longitudinal sections of maximally stretched muscle (50% greater than the resting muscle length). Immediately after dissection, the muscle was stretched and fixed in 4% formaldehyde for 30 min at 4°C and processed for cryoultramicrotomy as described by Griffiths et al. (1984) with modification. In brief, after fixation, muscles were cut into small strips (4–5-mm long × 1-mm wide) and transferred to 1.8 M sucrose/17% polyvinylpyrrolidone for overnight infusion. Muscle strips were trimmed further, mounted on cryopins, and snap-frozen in liquid nitrogen. Semi-thin (0.5–1.0 μm) sections were cut at −60°C using an Ultracut UCT ultramicrotome (Leica) equipped with an EM FCS cryochamber (Leica). Sections were suspended in 2.3 M sucrose, allowed to thaw, and placed on poly-O-lysine coated slides (Starfrost).

Immunostaining. Both thick (7 μm) and semi-thin (0.5–1.0 μm) sections were blocked overnight (4°C) in PBS Triton X-100 (0.05%) containing 0.2% fish gelatin and 2% BSA. The primary antibodies were applied for 60–90 min at RT, slides were washed with PBS, and secondary antibody was applied (60–90 min at RT). Double staining was performed by applying primary/secondary antibody pairs sequentially, i.e., the first primary and its secondary and then the second primary and its secondary. After washing to remove unbound secondary antibody, slides were mounted with Vectashield (Vector Laboratories) and viewed using a confocal laser scanning microscope (model TCS SP2; Leica) with an oil immersion 63× objective and analyzed with confocal software (Leica).

Antibodies

The polyclonal antibodies γ9d and α9d were directed against the exon 9d of the γ- and α-TM genes, respectively. The γ9d antibody is a polyclonal sheep antibody and detects isoforms Tm5NM1 and Tm5NM2 (Percival et al., 2004; Fig. 1 C), whereas α9d (originally WSα/9d) is a polyclonal antibody that detects Tm2, Tm3, Tm5a, Tm5b, and Tm6 from the α-TM gene and Tm1 from the β-TM gene (Schevzov et al., 1997; Fig. 1, A and B). Either a polyclonal rabbit or sheep α9d antibody was used depending on the species of the costaining antibody. CG3 is a mouse monoclonal that recognizes exon 1b of the γ-TM gene and detects all NS products (Fig. 1 C; Lin et al., 1988). The affinity purified γ-actin antibody is a polyclonal sheep antibody raised against the first 14 aa of cytoplasmic γ-actin (EEEIAALVID-NGSG). The commercially available mouse monoclonal 311 Tm antibody (Sigma-Aldrich), which recognizes Tms containing exon 1a sequence from the α-, β-, and γ-TM genes, was used. The α-actinin-2 antibody was a rabbit polyclonal antiserum was provided by A. Beggs (Children's Hospital, Boston, MA; North and Beggs, 1996). A rabbit polyclonal antibody recognizing the rod domain of dystrophin (Dys6-10) was provided by L. Kunkel (Children's Hospital). Slow fibers were detected with mAbs to the slow MHC isoform (undiluted BA-F8; Sigma-Aldrich) obtained from supernatants of hybridoma cultures (Borrione et al., 1988). Double staining with TRITC-labeled phalloidin (Sigma-Aldrich) was performed after the application of the primary antibody.

The following commercially available secondary antibodies were used: donkey anti–mouse HRP (Amersham Biosciences); donkey anti–rabbit HRP (Amersham Biosciences); donkey anti–sheep HRP (Jackson ImmunoResearch Laboratories); goat anti–rabbit FITC-labeled IgG (Sigma-Aldrich); goat anti–mouse Cy3 (Jackson ImmunoResearch Laboratories); and goat anti–rabbit Cy3 (Jackson ImmunoResearch Laboratories). The following Alexa-conjugated antibodies were also used (Molecular Probes): donkey anti–mouse IgG (L + H) Alexa 488; donkey anti–sheep IgG Alexa 488 and 555; goat anti–mouse IgM Alexa 488; goat anti–mouse IgG Alexa 488; and goat anti–rabbit IgG Alexa 488 and 555. All secondary antibodies were used at a 1:1,000 dilution.

Histopathological analysis

Mouse muscles were frozen as described above for immunohistochemistry. Sections were placed on poly-L-lysine precoated glass microscope slides, air dried, and stained with H&E or the modified Gomori-Trichrome method (Engel and Cunningham, 1963).

EM

Muscles were removed immediately after euthanasia and cut into very thin slices while immersed in modified Karnovski's fixative (2.5% glutaraldehyde/4% PFA in 1 M cacodylate buffer, pH 7.4). Samples were further fixed overnight in the same fixative and fixed after with 2% osmium tetroxide, dehydrated through an ascending series of ethanol, and embedded in Spurr's epoxy resin. Ultrathin sections were cut with a Reichert-Jung Ultracut ultramicrotome, double contrasted with uranyl acetate and lead citrate, and viewed and photographed with a BioTwin transmission electron microscope (model CM120; Philips).

Western blot analysis

Skeletal muscle tissue was homogenized in 20 vol of 10 mM Tris, pH 7.6/2% SDS/20 mM DTT by boiling followed by crushing with a pestle in a microtube. The samples were then solubilized by gentle sonication, boiled again, and spun at 12,000 rpm to remove insoluble particles. An equal volume of SDS-PAGE sample buffer (4% SDS, 12% glycerol, 2% mercaptoethanol, 0.01% Coomassie G-250, 50 mM Tris-Cl, pH 6.8) was added and the sample stored at −20°C. Equal volumes of protein (10 μg) were analyzed on 15% SDS-PAGE (with low 0.9% bis-acrylamide) gels with reference to a standard known amount of brain protein previously determined using a BCA protein assay kit (Pierce Chemical Co.) and volumes adjusted where necessary. Coomassie-stained gels were used to verify equal loading. Protein was transferred onto PVDF membranes (Millipore) at 80 V at 4°C. Blots were blocked in 5% skim milk at 4°C, washed in TBS, and incubated with the antibodies for 2 h at RT followed by three washes with TBS 0.05% Tween 20. HRP-labeled secondary antibody was added at 1:10,000 dilution for 1 h at RT. Excess antibody was removed with 4 × 20 min washes. Detection was performed using the Western lightning chemiluminescence detection system (PerkinElmer) on Biomax X-ray film (Kodak) for varying times (2–60 min).

Serum creatine kinase measurement

Blood was taken from 6-mo-old anesthetized (halothane) WT and Tm3 mice by cardiac puncture. Serum creatine kinase was measured using a commercial kit and a serum quality control sample (Trace/DMA; Thermo Electron).

References

Arai, A., J.A. Spencer, and E.N. Olson. 2002. STARS, a striated muscle activator of Rho signaling and serum response factor-dependent transcription. *J. Biol. Chem.* 277:24453–24459.

Bang, M.L., R.E. Mudry, A.S. McElhinny, K. Trombitas, A.J. Geach, R. Yamasaki, H. Sorimachi, H. Granzier, C.C. Gregorio, and S. Labeit. 2001. Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. *J. Cell Biol.* 153:413–427.

Banker, B.Q., and A.G. Engel. 1994. Basic Reactions of Muscle. *In* Myology. A.G. Engel and Franzini-Armstrong C., editors. McGraw-Hill, Inc., New York. 832–888.

Bittner, R.E., L.V. Anderson, E. Burkhardt, R. Bashir, E. Vafiadaki, S. Ivanova, T. Raffelsberger, I. Maerk, H. Hoger, M. Jung, et al. 1999. Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. *Nat. Genet.* 23:141–142.

Borrione, A.C., A.M. Zanellato, L. Saggin, M. Mazzoli, G. Azzarello, and S. Sartore. 1988. Neonatal myosin heavy chains are not expressed in Ni-induced rat rhabdomyosarcoma. *Differentiation.* 38:49–59.

Bottinelli, R., D.A. Coviello, C.S. Redwood, M.A. Pellegrino, B.J. Maron, P. Spirito, H. Watkins, and C. Reggiani. 1998. A mutant tropomyosin that causes hypertrophic cardiomyopathy is expressed in vivo and associated with an increased calcium sensitivity. *Circ. Res.* 82:106–115.

Boyd, J., J.I. Risinger, R.W. Wiseman, B.A. Merrick, J.K. Selkirk, and J.C. Barrett. 1995. Regulation of microfilament organization and anchorage-independent growth by tropomyosin 1. *Proc. Natl. Acad. Sci. USA.* 92:11534–11538.

Bryce, N.S., G. Schevzov, V. Ferguson, J.M. Percival, J.J. Lin, F. Matsumura, J.R.

Bamburg, P.L. Jeffrey, E.C. Hardeman, P. Gunning, and R.P. Weinberger. 2003. Specification of actin filament function and molecular composition by tropomyosin isoforms. *Mol. Biol. Cell.* 14:1002–1016.

Capetanaki, Y. 2002. Desmin cytoskeleton: a potential regulator of muscle mitochondrial behavior and function. *Trends Cardiovasc. Med.* 12:339–348.

Clark, K.A., A.S. McElhinny, M.C. Beckerle, and C.C. Gregorio. 2002. Striated muscle cytoarchitecture: an intricate web of form and function. *Annu. Rev. Cell Dev. Biol.* 18:637–706.

Coghill, I.D., S. Brown, D.L. Cottle, M.J. McGrath, P.A. Robinson, H.H. Nandurkar, J.M. Dyson, and C.A. Mitchell. 2003. FHL3 is an actin-binding protein that regulates alpha-actinin-mediated actin bundling: FHL3 localizes to actin stress fibers and enhances cell spreading and stress fiber disassembly. *J. Biol. Chem.* 278:24139–24152.

Craig, S.W., and J.V. Pardo. 1983. Gamma actin, spectrin, and intermediate filament proteins colocalize with vinculin at costameres, myofibril-to-sarcolemma attachment sites. *Cell Motil.* 3:449–462.

Dalby-Payne, J.R., E.V. O'Loughlin, and P. Gunning. 2003. Polarization of specific tropomyosin isoforms in gastrointestinal epithelial cells and their impact on CFTR at the apical surface. *Mol. Biol. Cell.* 14:4365–4375.

Dalkilic, I., and L.M. Kunkel. 2003. Muscular dystrophies: genes to pathogenesis. *Curr. Opin. Genet. Dev.* 13:231–238.

Dufour, C., R.P. Weinberger, and P. Gunning. 1998a. Tropomyosin isoform diversity and neuronal morphogenesis. *Immunol. Cell Biol.* 76:424–429.

Dufour, C., R.P. Weinberger, G. Schevzov, P.L. Jeffrey, and P. Gunning. 1998b. Splicing of two internal and four carboxyl-terminal alternative exons in nonmuscle tropomyosin 5 pre-mRNA is independently regulated during development. *J. Biol. Chem.* 273:18547–18555.

Engel, W.K., and G.G. Cunningham. 1963. Rapid examination of muscle tissue. An improved trichrome method for fresh-frozen biopsy sections. *Neurology.* 13:919–923.

Erdelyi, M., A.M. Michon, A. Guichet, J.B. Glotzer, and A. Ephrussi. 1995. Requirement for *Drosophila* cytoplasmic tropomyosin in oskar mRNA localization. *Nature.* 377:524–527.

Ervasti, J.M. 2003. Costameres: the Achilles' heel of Herculean muscle. *J. Biol. Chem.* 278:13591–13594.

Fanning, A.S., J.S. Wolenski, M.S. Mooseker, and J.G. Izant. 1994. Differential regulation of skeletal muscle myosin-II and brush border myosin-I enzymology and mechanochemistry by bacterially produced tropomyosin isoforms. *Cell Motil. Cytoskeleton.* 29:29–45.

Flicker, P.F., G.N. Phillips Jr., and C. Cohen. 1982. Troponin and its interactions with tropomyosin. An electron microscope study. *J. Mol. Biol.* 162:495–501.

Gimona, M., J.A. Kazzaz, and D.M. Helfman. 1996. Forced expression of tropomyosin 2 or 3 in v-Ki-ras-transformed fibroblasts results in distinct phenotypic effects. *Proc. Natl. Acad. Sci. USA.* 93:9618–9623.

Griffiths, G., A. McDowall, R. Back, and J. Dubochet. 1984. On the preparation of cryosection for immunocytochemistry. *J. Ultrastruct. Res.* 89:65–78.

Gunning, P., M. Gordon, R. Wade, R. Gahlmann, C.S. Lin, and E. Hardeman. 1990. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulatory compensation control mechanism. *Dev. Biol.* 138:443–453.

Gunning, P., E. Hardeman, P. Jeffrey, and R. Weinberger. 1998a. Creating intracellular structural domains: spatial segregation of actin and tropomyosin isoforms in neurons. *Bioessays.* 20:892–900.

Gunning, P., R. Weinberger, P. Jeffrey, and E. Hardeman. 1998b. Isoform sorting and the creation of intracellular compartments. *Annu. Rev. Cell Dev. Biol.* 14:339–372.

Guy, P.M., D.A. Kenny, and G.N. Gill. 1999. The PDZ domain of the LIM protein enigma binds to beta-tropomyosin. *Mol. Biol. Cell.* 10:1973–1984.

Hall, Z.W., B.W. Lubit, and J.H. Schwartz. 1981. Cytoplasmic actin in postsynaptic structures at the neuromuscular junction. *J. Cell Biol.* 90:789–792.

Hannan, A.J., P. Gunning, P.L. Jeffrey, and R.P. Weinberger. 1998. Structural compartments within neurons: developmentally regulated organization of microfilament isoform mRNA and protein. *Mol. Cell. Neurosci.* 11:289–304.

Heimann, K., J.M. Percival, R. Weinberger, P. Gunning, and J.L. Stow. 1999. Specific isoforms of actin-binding proteins on distinct populations of Golgi-derived vesicles. *J. Biol. Chem.* 274:10743–10750.

Herman, I.M. 1993. Actin isoforms. *Curr. Opin. Cell Biol.* 5:48–55.

Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. Manipulating the Mouse Embryo. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 332 pp.

Hook, J., F. Lemckert, H. Qin, G. Schevzov, and P. Gunning. 2004. Gamma tropomyosin gene products are required for embryonic development. *Mol. Cell.*

Biol. 24:2318–2323.

Ishikawa, R., S. Yamashiro, and F. Matsumura. 1989a. Annealing of gelsolin-severed actin fragments by tropomyosin in the presence of Ca^{2+}. Potentiation of the annealing process by caldesmon. *J. Biol. Chem.* 264:16764–16770.

Ishikawa, R., S. Yamashiro, and F. Matsumura. 1989b. Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin. Potentiation of protective ability of tropomyosins by 83-kDa nonmuscle caldesmon. *J. Biol. Chem.* 264:7490–7497.

Kislauskis, E.H., X. Zhu, and R.H. Singer. 1997. β-Actin messenger RNA localization and protein synthesis augment cell motility. *J. Cell Biol.* 136:1263–1270.

Kojima, H., A. Ishijima, and T. Yanagida. 1994. Direct measurement of stiffness of single actin filaments with and without tropomyosin by in vitro nanomanipulation. *Proc. Natl. Acad. Sci. USA.* 91:12962–12966.

Kumar, A., K. Crawford, L. Close, M. Madison, J. Lorenz, T. Doetschman, S. Pawlowski, J. Duffy, J. Neumann, J. Robbins, et al. 1997. Rescue of cardiac alpha-actin-deficient mice by enteric smooth muscle gamma-actin. *Proc. Natl. Acad. Sci. USA.* 94:4406–4411.

Lehman, W., V. Hatch, V. Korman, M. Rosol, L. Thomas, R. Maytum, M.A. Geeves, J.E. Van Eyk, L.S. Tobacman, and R. Craig. 2000. Tropomyosin and actin isoforms modulate the localization of tropomyosin strands on actin filaments. *J. Mol. Biol.* 302:593–606.

Lin, J.J., T.E. Hegmann, and J.L. Lin. 1988. Differential localization of tropomyosin isoforms in cultured nonmuscle cells. *J. Cell Biol.* 107:563–572.

Lin, J.J., K.S. Warren, D.D. Wamboldt, T. Wang, and J.L. Lin. 1997. Tropomyosin isoforms in nonmuscle cells. *Int. Rev. Cytol.* 170:1–38.

Lubit, B.W. 1984. Association of beta-cytoplasmic actin with high concentrations of acetylcholine receptor (AChR) in normal and anti-AChR-treated primary rat muscle cultures. *J. Histochem. Cytochem.* 32:973–981.

Matsumura, F., and S. Yamashiro-Matsumura. 1985. Purification and characterization of multiple isoforms of tropomyosin from rat cultured cells. *J. Biol. Chem.* 260:13851–13859.

Matsumura, F., S. Yamashiro-Matsumura, and J.J. Lin. 1983. Isolation and characterization of tropomyosin-containing microfilaments from cultured cells. *J. Biol. Chem.* 258:6636–6644.

Milner, D.J., M. Mavroidis, N. Weisleder, and Y. Capetanaki. 2000. Desmin cytoskeleton linked to muscle mitochondrial distribution and respiratory function. *J. Cell Biol.* 150:1283–1298.

Miyado, K., M. Kimura, and S. Taniguchi. 1996. Decreased expression of a single tropomyosin isoform, TM5/TM30nm, results in reduction in motility of highly metastatic B16-F10 mouse melanoma cells. *Biochem. Biophys. Res. Commun.* 225:427–435.

Moreira, E.S., T.J. Wiltshire, G. Faulkner, A. Nilforoushan, M. Vainzof, O.T. Suzuki, G. Valle, R. Reeves, M. Zatz, M.R. Passos-Bueno, and D.E. Jenne. 2000. Limb-girdle muscular dystrophy type 2G is caused by mutations in the gene encoding the sarcomeric protein telethonin. *Nat. Genet.* 24:163–166.

Muthuchamy, M., K. Pieples, P. Rethinasamy, B. Hoit, I.L. Grupp, G.P. Boivin, B. Wolska, C. Evans, R.J. Solaro, and D.F. Wieczorek. 1999. Mouse model of a familial hypertrophic cardiomyopathy mutation in alpha-tropomyosin manifests cardiac dysfunction. *Circ. Res.* 85:47–56.

Nakata, T., Y. Nishina, and H. Yorifuji. 2001. Cytoplasmic gamma actin as a Z-disc protein. *Biochem. Biophys. Res. Commun.* 286:156–163.

North, K.N., and A.H. Beggs. 1996. Deficiency of a skeletal muscle isoform of alpha-actinin (alpha-actinin-3) in merosin-positive congenital muscular dystrophy. *Neuromuscul. Disord.* 6:229–235.

North, A.J., M. Gimona, Z. Lando, and J.V. Small. 1994. Actin isoform compartments in chicken gizzard smooth muscle cells. *J. Cell Sci.* 107:445–455.

Pardo, J.V., M.F. Pittenger, and S.W. Craig. 1983. Subcellular sorting of isoactins: selective association of gamma-actin with skeletal muscle mitochondria. *Cell.* 32:1093–1103.

Palmer, S., N. Groves, A. Schindeler, T. Yeoh, C. Biben, C.C. Wang, D.B. Sparrow, L. Barnett, N.A. Jenkins, N.G. Copeland, et al. 2001. The small muscle-specific protein Csl modifies cell shape and promotes myocyte fusion in an insulin-like growth factor 1-dependent manner. *J. Cell Biol.* 153:985–998.

Percival, J.M., G. Thomas, T.A. Cock, E.M. Gardiner, P.L. Jeffrey, J.J. Lin, R.P. Weinberger, and P. Gunning. 2000. Sorting of tropomyosin isoforms in synchronised NIH 3T3 fibroblasts: evidence for distinct microfilament populations. *Cell Motil. Cytoskeleton.* 47:189–208.

Percival, J.M., J.A.I. Hughes, D.L. Brown, G. Schevzov, K. Heimann, B. Vrhovski, N. Bryce, J.L. Stow, and P.W. Gunning. 2004. Targeting of a tropomyosin isoform to short microfilaments associated with the Golgi complex. *Mol. Biol. Cell.* 15:268–280.

Pittenger, M.F., J.A. Kazzaz, and D.M. Helfman. 1994. Functional properties of

non-muscle tropomyosin isoforms. *Curr. Opin. Cell Biol.* 6:96–104.

Pittenger, M.F., A. Kistler, and D.M. Helfman. 1995. Alternatively spliced exons of the beta tropomyosin gene exhibit different affinities for F-actin and effects with nonmuscle caldesmon. *J. Cell Sci.* 108:3253–3265.

Prasad, G.L., R.A. Fuldner, and H.L. Cooper. 1993. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the ras oncogene. *Proc. Natl. Acad. Sci. USA.* 90:7039–7043.

Richard, I., O. Broux, V. Allamand, F. Fougerousse, N. Chiannilkulchai, N. Bourg, L. Brenguier, C. Devaud, P. Pasturaud, C. Roudaut, et al. 1995. Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell.* 81:27–40.

Ronnov-Jessen, L., and O.W. Petersen. 1996. A function for filamentous α-smooth muscle actin: retardation of motility in fibroblasts. *J. Cell Biol.* 134:67–80.

Rybakova, I.N., J.R. Patel, and J.M. Ervasti. 2000. The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. *J. Cell Biol.* 150:1209–1214.

Schevzov, G., C. Lloyd, and P. Gunning. 1992. High level expression of transfected β- and γ-actin genes differentially impacts on myoblast cytoarchitecture. *J. Cell Biol.* 117:775–785.

Schevzov, G., P. Gunning, P.L. Jeffrey, C. Temm-Grove, D.M. Helfman, J.J. Lin, and R.P. Weinberger. 1997. Tropomyosin localization reveals distinct populations of microfilaments in neurites and growth cones. *Mol. Cell. Neurosci.* 8:439–454.

Seward, D.J., J.C. Haney, M.A. Rudnicki, and S.J. Swoap. 2001. bHLH transcrip-

tion factor MyoD affects myosin heavy chain expression pattern in a muscle-specific fashion. *Am. J. Physiol. Cell Physiol.* 280:C408–C413.

Temm-Grove, C.J., B.M. Jockusch, R.P. Weinberger, G. Schevzov, and D.M. Helfman. 1998. Distinct localizations of tropomyosin isoforms in LLC-PK1 epithelial cells suggests specialized function at cell-cell adhesions. *Cell Motil. Cytoskeleton.* 40:393–407.

Thierfelder, L., H. Watkins, C. MacRae, R. Lamas, W. McKenna, H.P. Vosberg, J.G. Seidman, and C.E. Seidman. 1994. Alpha-tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell.* 77:701–712.

Tsukamoto, Y., T. Senda, T. Nakano, C. Nakada, T. Hida, N. Ishiguro, G. Kondo, T. Baba, K. Sato, M. Osaki, et al. 2002. Arpp, a new homolog of carp, is preferentially expressed in type 1 skeletal muscle fibers and is markedly induced by denervation. *Lab. Invest.* 82:645–655.

von Arx, P., S. Bantle, T. Soldati, and J.C. Perriard. 1995. Dominant negative effect of cytoplasmic actin isoproteins on cardiomyocyte cytoarchitecture and function. *J. Cell Biol.* 131:1759–1773.

Weinberger, R., G. Schevzov, P. Jeffrey, K. Gordon, M. Hill, and P. Gunning. 1996. The molecular composition of neuronal microfilaments is spatially and temporally regulated. *J. Neurosci.* 16:238–252.

Wieczorek, D.F., M. Periasamy, G.S. Butler-Browne, R.G. Whalen, and B. Nadal-Ginard. 1985. Co-expression of multiple myosin heavy chain genes, in addition to a tissue-specific one, in extraocular musculature. *J. Cell Biol.* 101:618–629.