The existence of a feedback mechanism regulating the precise amounts of muscle structural proteins, such as actin and the actin-associated protein tropomyosin (Tm), in the sarcomeres of striated muscles is well established. However, the regulation of nonmuscle or cytoskeletal actin and Tms in nonmuscle cell structures has not been elucidated. Unlike the thin filaments of striated muscles, the actin cytoskeleton in nonmuscle cells is intrinsically dynamic. Given the differing requirements for the structural integrity of the actin thin filaments of the sarcomere compared with the requirement for dynamicity of the actin cytoskeleton in nonmuscle cells, we postulated that different regulatory mechanisms govern the expression of sarcomeric versus cytoskeletal Tms, as key regulators of the properties of the actin cytoskeleton. Comprehensive analyses of tissues from transgenic and knock-out mouse lines that overexpress the cytoskeletal Tms, as key regulators of the properties of the actin cytoskeleton (known as cytoskeletal Tms), whereas three Tm isoforms (striated muscle or sarcomeric Tms) are exclusively expressed in striated muscle and associate with actin in the thin filament of the sarcomere (13).

Actin microfilaments are present in a variety of cellular structures that are specialized for different functions (1). In muscle cells, actin filaments are arranged into the thin filaments of sarcomeres to provide contractile force. Proper functioning of the sarcomere requires an invariant organization of this structure. A mechanism is in place that maintains strict stoichiometric expression of the components of the sarcomere, such as actin and Tms, and thus sarcomeric integrity. In contrast, actin microfilaments in nonmuscle cells are involved in a wide range of cellular architectures and functions, including motility, membrane ruffling, adhesion, cytokinesis, and transport. The diverse activities of the actin microfilaments involved in these cellular processes is made possible due to the dynamic nature of the actin cytoskeleton, where actin filaments undergo rapid assembly and disassembly through monomeric to filamentous actin conversion.

Among the extensive array of proteins that interact directly or indirectly with actin (2) and regulate the dynamics and assembly of actin filaments, the Tms play an essential role. Tms stabilize actin filaments by modulating the interaction of actin-binding proteins responsible for the regulation of actin dynamics (3–8). The majority of the ~40 mammalian Tm isoforms (9–12) are found associated with actin filaments of the cytoskeleton (known as cytoskeletal Tms), whereas three Tm isoforms (striated muscle or sarcomeric Tms) are exclusively expressed in striated muscle and associate with actin in the thin filament of the sarcomere (13).

A number of studies have established the existence of a feedback mechanism in striated muscles such that forced overexpression or knockdown of genes encoding sarcomere-associated contractile proteins results in translational compensation that maintains a fixed amount for a given sarcomeric protein (14–24). This is evident in the regulation of the sarcomeric Tms in cardiac muscle: α-Tm (major isoform) and β-Tm (minor isoform) (25). Hemizygous knock-out of striated α-Tm results in reduced levels of α-Tm mRNA; nevertheless, normal levels of striated α-Tm protein are maintained (26, 27). Correspondingly, overexpression of β-Tm in the hearts of transgenic mice elicits a reduction in the levels of α-Tm. Thus, homeostasis of the amount of sarcomeric Tm is maintained (15). Based on these studies, it is postulated that translational compensation ensures that the level of the total sarcomeric protein pool remains unaltered, hence maintaining the strict protein stoichiometry of the sarcomere (14).

We hypothesized that in contrast to the requirement for a feedback mechanism to maintain a strict protein stoichiometry in the muscle sarcomere, the dynamic nature of the nonmuscle actin cytoskeleton requires a more flexible mechanism. We envisaged a simple mechanism whereby the supply of cytoskel-
et al. Tms might be limiting. Thus the altered expression of limiting cytoskeletal Tm isoforms could provide a mechanism to regulate the form and dynamics of actin cytoskeletal structures. Through analysis of cytoskeletal Tm expression in transgenic and knock-out mice, we demonstrate that cytoskeletal Tms are not regulated by a feedback mechanism. We show that sarcomeric and cytoskeletal Tms in striated muscle cells are regulated by different mechanisms. Overexpression of muscle β-Tm in the heart causes the expected decrease in α-Tm expression (15) yet has no impact on the expression of Tm4, a cytoskeletal isoform endogenously expressed in cardiac muscle cells. We show that an increase in the level of Tm5NM1 in the growth cone of primary neurons leads to an increased level of actin polymer. This divergent regulation of the Tm isoforms in striated versus nonmuscle cells provides a mechanism to regulate actin microfilament structures with clearly distinct functional requirements.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The primary rabbit polyclonal antibody, WD4/9d, that detects Tm4 encoded by the β-Tm gene (28), was used at 1:400. Mouse monoclonal antibodies were used at the following dilutions: α-actin at 1:100 (clone EA-53; Sigma), α-tubulin at 1:1000 (clone DM 1A; Sigma), β-actin at 1:1000 (clone AC-74; Sigma) (29), CG3 (IgM class) at 1:250 (30), LC1 at 1:250 (31), C4 total actin at 1:500 (a kind gift of Dr. Jim Lessard) (32), antisarcomeric tropomyosin at 1:50 (clone CH1, T-9283; Sigma) (33). Primary sheep polyclonal antibodies were used at the following dilutions: α/9d at 1:400 (34), γ/9a at 1:100, γ/c at 1:100 (35), γ/9d at 1:100 (36), α-actin at 1:1000 (34). Secondary antibodies were used Alexa Fluor 488 donkey anti-mouse IgG (H + L) conjugate, Cy3 donkey anti-sheep IgG (H + L) and Cy3 donkey anti-rabbit IgG (H + L) (1:1000 dilution) (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA). Nuclei were detected with 4',6-diamidino-2-phenylindole (300 nm), and F-actin was detected with Alexa Fluor 647 Phalloidin (Molecular Probes).

**Primary Mouse Embryonic Fibroblasts (MEFs) and Hippocampal Neuron Cultures**—Isolation and culture of MEFs from nontransgenic (NTG), TG5/52 and Tm5NM1/NM2 knock-out mice was carried out as described by Schevzov et al. (34). In brief, 13.5-day-old embryos were isolated, and limbs, heads, and internal organs were removed. Trunks were incubated in 0.25% trypsin/EDTA (Invitrogen) for 15 min at 37 °C and dissociated by passage through a Pasteur pipette. Large pieces of cellular debris were allowed to settle, and cells remaining in suspension were plated in Dulbecco’s modified Eagle’s medium (high glucose), 2 mm l-glutamine (Invitrogen), 10% fetal calf serum (Invitrogen), 0.1 mm β-mercaptoethanol (Sigma), 50 units/ml penicillin, and 50 mg/ml streptomycin. Cells were maintained at 37 °C in a humidified 5% CO2 incubator. Hippocampal cell cultures were prepared as described previously (37, 38) with the following changes. Dissociated cultures were prepared from embryonic day 16.5 mouse hippocampi (39) from Tg5/52 and NTG control animals. After dissociation, Tg5/52 and NTG cells were mixed at a ratio of 1:1, plated at a density of 2500 cells/cm2 onto poly-γ-lysine-coated glass coverslips (13-mm round number 1 glass coverslips; Menzel-Glauser, Braunschweig, Germany) in 24-well plates and cultured in 500 μl of Dulbecco’s modified Eagle’s medium (Invitrogen), 10% fetal bovine serum (Hyclone; Thermo Fischer Scientific Inc.) at 37 °C in a humidified 5% CO2 incubator. Two hours after plating, medium was changed to Neurobasal medium containing B27 supplement, 0.5 mm Glutamax (Invitrogen).

**Generation of Transgenic and Knock-out Mice**—All animal experiments were performed in accordance with institutional and National Health and Medical Research Council of Australia guidelines. Generation of the Tm3 and Tm5NM1 transgenic lines has been described previously (6, 40). In brief, the rat Tm3 cDNA that encodes a protein identical to the mouse and the human Tm5NM1 cDNA that differs from the mouse by one amino acid were cloned into the human β-actin expression vector (41). The targeting vector used to delete exon 9d-containing products from the γ-Tm gene and hence generate the Tm5NM1/NM2 knock-out mouse line is described in detail elsewhere.β-Tm transgenic mice in which heart-specific expression of β-Tm is driven with the cardiac α-MHC promoter have been described previously (15).

**Gel Electrophoresis and Western Blot Analysis**—Tissues from two NTG, five TG3/66, two TG3/70, and two Tg5/52 mice, ~3 months old, were used. Tissues were excised, frozen in liquid nitrogen, and dissociated in a sufficient volume of 50 mls Tris, pH 7.5, using a Polytron blender. Proteins were solubilized in SDS solubilization buffer (0.125 M Tris, pH 6.8, 0.5% SDS, 5% glycerol, 5% mercaptoethanol, 0.005% bromphenol blue), and the protein concentration was determined using a BCA protein assay kit (Pierce). Hearts from β-Tm transgenic mice, NTG control mice, and frozen embryonic stem (ES) and MEF cell pellets were processed for protein in a similar manner. Proteins were separated by SDS-PAGE using 12.5% acrylamide, 0.1% bisacrylamide (42) and transferred to Immobilon-P polyvinylidene difluoride (Millipore Corp., Bedford, MA) for 1 h at 30 V (43), and blots were incubated in 5% low fat skim milk in TBS (100 mm Tris-HCl, pH 7.5, 150 mm NaCl). Blots were incubated with primary and secondary antibodies (diluted in TBS) (anti-rabbit, anti-sheep, or anti-mouse Ig-conjugated horseradish peroxidase) (Amersham Biosciences) for 1 h each followed by four 15-min washes with TTBS (TBS with 0.05% Tween 20) after each antibody incubation. Blots were developed with the Western Lighting chemiluminescence reagent (PerkinElmer Life Sciences) and exposed to Fuji x-ray film (FUJIFILM Australia). Each blot shown in Figs. 1–4 and 7 is representative of 3–5 independent Western blots. Prestained molecular weight markers were used (BenchMarkTTM prestained protein ladder; Invitrogen).

**Statistical Analysis**—For the quantitation of Western blots, several film exposures of each blot were made and scanned at 300 dots/inch, and the signal intensities of bands were quantified using NIH Image. Volume analysis with object background correction was applied. Signal intensity in each lane was expressed relative to α-tubulin staining of the same blot to control for protein loading differences. The statistical difference was estimated by Student’s t test, and the criterion for statistical

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significance was set at \( p < 0.05 \). Intensity of phalloidin staining and growth cone size measurements was made using Meta-Morph software (Molecular Devices Ltd., Victoria, Australia). Surface area measurement of the growth cones was performed by tracing the perimeter of the growth cone, and the proximal region of the growth cone was delineated at the point where it first splayed from the axon. Statistical analysis was performed using SPSS version 13, and groups were compared using non-parametric analysis (the Mann-Whitney \( U \) test). All experimental values are expressed as \( \pm \) S.D.

**RESULTS**

**Overexpression of Cytoskeletal Tms Does Not Elicit a Feedback Response**—To investigate the hypothesis that cytoskeletal Tm isoforms are not subject to feedback regulation, we employed transgenic mouse models that overexpress two different cytoskeletal Tm isoforms encoded by different Tm genes. Tm3 is a high molecular weight isoform encoded by the \( \alpha \)-Tm gene and is predominantly expressed in the stomach of NTG (Fig. 1F). Tm5NM1 is a low molecular weight isoform encoded by the \( \gamma \)-Tm gene and is ubiquitously expressed (34, 40). We confirmed that levels of Tm3 and Tm5NM1 are significantly increased in transgenic tissues of the respective mouse line (Fig. 1). Tm5NM1 is a low molecular weight isoform encoded by the \( \gamma \)-Tm gene and is ubiquitously expressed (34, 40). We confirmed that levels of Tm3 and Tm5NM1 are significantly increased in transgenic tissues of the respective mouse line (Fig. 1). Tm5NM1 is a low molecular weight isoform encoded by the \( \gamma \)-Tm gene and is ubiquitously expressed (34, 40). We confirmed that levels of Tm3 and Tm5NM1 are significantly increased in transgenic tissues of the respective mouse line (Fig. 1). Tm5NM1 is a low molecular weight isoform encoded by the \( \gamma \)-Tm gene and is ubiquitously expressed (34, 40). We confirmed that levels of Tm3 and Tm5NM1 are significantly increased in transgenic tissues of the respective mouse line (Fig. 1).
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| Antibody name | Tm gene | Tm isoform recognition | References |
|---------------|---------|------------------------|------------|
| α/9d          | α, β    | Tm6, -1, -2, -3, 5a, 5b | 53         |
| γ/9d          | γ       | Tm5NM1 and -NM2        | 36         |
| CG3           | γ       | All products            | 30         |
| WD4/9d        | δ       | Tm4                    | 28         |
| LC1           | γ       | Human Tm5NM1           | 31         |
| CH1           | α, β, γ | Muscle α, β, γ-Tms     | 33         |

FIGURE 2. Overexpression of either Tm3 or Tm5NM1 has no impact on the expression of other cytoskeletal Tm isoforms. 20 µg of total cellular protein was isolated from two representative tissues, brain and lung, and analyzed by low bisacrylamide SDS-PAGE. Western blots were probed with WD4/9d (A and D), CG3 (B and E), and α/9d (C). Densitometric analysis confirmed no significant changes in expression of the Tm isoform Tm6, Tm1, Tm2, Tm5a, Tm5b, or Tm4 or Tm isoforms encoded by the γ-Tm gene (supplemental Fig. S1). Similarly, no significant changes in the level of expression of Tm6, Tm1, Tm2, Tm5a, Tm5b, or Tm4 were observed in Tm5NM1 transgenic tissues (Fig. 2, C–E, and supplemental Fig. S2, A–E). The increased amount of cytoskeletal γ-Tm isoforms in the Tm5NM1 transgenic tissues, detected with the total γ-Tm antibody (CG3), is due to the exogenously expressed human Tm5NM1 (Fig. 2E and supplemental Fig. S2F) and was predominantly observed in the brain, kidney, and stomach, mirroring the strong transgene expression in these tissues established earlier (Fig. 1C). In conclusion, Tm3 and Tm5NM1 proteins can stably accumulate in tissues if their supplies are increased, demonstrating that cytoskeletal protein pools do not appear to be saturated. In addition, since no compensating decreases in expression of endogenous isoforms are observed, the total cytoskeletal Tm pool is not fixed.

In a complementary approach, we investigated the profile of cytoskeletal Tm expression in cells derived from heterozygous knock-out mice in which Tm5NM1 and Tm5NM2, the two major cytoskeletal isoforms from the γ-Tm gene, are deleted.3 Both ES cell clones and mouse embryo fibroblasts (MEFs) derived from heterozygous knock-out mouse embryos display an ~50% reduction in the level of Tm5NM1 protein (Fig. 3A). Separate analysis with an antibody that detects all γ-Tm gene products (CG3) similarly reveals an ~50% reduction in protein (Fig. 3B). Importantly, no compensating change in the expression of other cytoskeletal Tms is observed in either the ES or MEF cells (Fig. 3, C and D). Densitometry was carried out on an average of three sets of samples with identical results (data not shown). These results lead us to conclude that unlike sarcomere-associated Tms, there is a direct correlation between cytoskeletal Tm gene expression and protein levels and an absence of translational compensation.

Cytoskeletal Tm Isoforms Are Regulated Independently of Muscle Tms in the Heart—We considered the possibility that the absence of translational compensation in response to forced overexpression or knock-down of cytoskeletal Tms may simply represent different regulatory processes operating in muscle and nonmuscle tissues. To directly test this possibility, we examined the regulation of cytoskeletal Tms within striated muscle. Our data demonstrate that overexpression of Tm3 or Tm5NM1 has no effect on the levels of other cytoskeletal Tm isoforms in transgenic heart muscle (Fig. 4, A, C, and D, right panels). Thus, endogenous cytoskeletal Tms fail to respond to elevated expression of transgene-encoded cytoskeletal Tms in both striated muscle and nonmuscle tissues.
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Next we investigated the regulation of cytoskeletal Tms in heart muscle overexpressing β-Tm, a system in which the sarcomere-associated Tm feedback mechanism is activated (15). Confirming previous findings, overexpression of β-Tm in the heart leads to a corresponding reduction in the expression of α-Tm (15) (Fig. 4E, left); however, there was no impact on the expression of cytoskeletal Tm isoforms (Fig. 4, A, C, and D, left panels). In order to confirm that cytoskeletal Tms are indeed co-expressed with muscle α-Tm in cardiac cells within the heart, we determined the localization of a representative cytoskeletal Tm, Tm4, by immunofluorescence detection. As shown in Fig. 5, Tm4 localizes near the Z-line, delineated by α-actinin (Fig. 5C), and is also present in filaments that run along the length of the cardiac cells (Fig. 5C, arrow). A similar pattern of Tm4 staining has recently been documented in skeletal muscle (45). Finally, we note that no significant changes in expression of the predominant mouse heart Tm isoform, α-Tm, were seen following the transgenic overexpression of Tm3 or Tm5NM1 (Fig. 4E, right). Together, these data indicate that the regulation of the cytoskeletal Tms is the same in either muscle-derived or nonmuscle tissue and is distinct from the compensatory mechanism regulating sarcomeric Tm expression.

Ectopically Expressed Cytoskeletal Tm Localizes to the Same Structures as the Endogenous Tm Isoform—Collectively, our data suggest that the supply of cytoskeletal Tm may be limiting for the formation of Tm-containing actin filaments. Accordingly, the expectation is that exogenous cytoskeletal Tm protein in the transgenic tissues should be incorporated into the same structures as those observed in wild-type tissues. We examined the distribution of Tm5NM1 in both wild-type and transgene-derived MEFs and in soleus muscle derived from the transgenic Tm5NM1 mouse model, since we can specifically detect the transgenic human Tm5NM1 protein expression using a human-specific antibody (LC1). Both endogenous and exogenous Tm5NM1 are present in the stress fibers within the midbody of the cell (Fig. 6, A and D, arrows) and to a lesser extent within the lamellipodia (Fig. 6, A and D, arrowheads) of MEFs. Similarly, ectopically expressed Tm5NM1 sorts to the same intracellular compartment within the sarcomere as the endogenous isoform (Fig. 6, G and J). Longitudinal sections of soleus muscle treated with γ/9d or LC1 and α-actinin antibodies show that both endogenous Tm5NM1 (Fig. 6, G–I) and exogenous Tm5NM1 (Fig. 6, J–L) localize to the Z-line adjacent compartment (Z-LAC) (44).

Actin Protein Levels Are Unaltered following Overexpression of Tm3 and Tm5NM1—We considered the possibility that actin expression might be impacted upon following overexpression of Tm3 or Tm5NM1. An actin antibody that detects both nonmuscle and muscle actin (32) was used to determine total actin expression, and we found that the levels of total actin remained relatively unchanged in transgenic tissues (Fig. 7, A and B, and supplemental Fig. S3). Moreover, no changes were seen in the levels of β-actin or γ-actin in transgenic tissues (data not shown). This result is in agreement with the recent finding that overexpression of the cytoskeletal isoforms Tm5NM1 and TmBr3 in a neuroepithelium cell line, B35, did not alter expression of actin (6). Therefore, overexpression of cytoskeletal Tm isoforms does not affect the expression of either actin or other cytoskeletal Tm isoforms.

Overexpression of Tm5NM1 Increases the F-actin Pool—Tm has been classified as a microfilament-stabilizing actin-binding protein (13). Although total actin protein levels remained unaltered following Tm overexpression, it is possible that elevated Tm could drive an increase in the levels of actin polymer. We have tested this in neuronal growth cones derived from Tm5NM1 transgenic mice. Primary hippocampal neurons isolated from the TG5/52 mice exhibit an enrichment of the exogenously expressed Tm5NM1 (detected with the LC1 antibody) in growth cones (Fig. 8A). The growth cones from hippocampal neurons are significantly larger (Fig. 8D, p < 0.005). This enrichment of Tm5NM1 in the growth cone corresponds with an increase in phallolidin staining (Fig. 8, A–C). Total phallolidin staining in the TG5/52 largest growth cones is significantly greater relative to that seen in the largest growth cones from NTG neurons (Fig. 8E; p < 0.0005). Additionally, the mean pixel phallolidin intensity within the largest TG5/52 growth cones is also significantly greater (Fig. 8F; p < 0.005). We conclude that increased cytoskeletal Tm levels can drive increased actin polymer levels.

DISCUSSION

In this study, we demonstrate that the cytoskeleton-associated Tms are subject to a different regulatory mechanism than
the sarcomere-associated Tms. Importantly, this is true for the cytoskeletal Tms present in both muscle and nonmuscle cells. Thus, the divergent regulation of the two populations of Tm isoforms is likely to be intrinsic to functional differences between the sarcomere and the cytoskeleton. We hypothesize that the absence of a feedback mechanism to regulate cytoskeletal Tm expression provides the necessary flexibility of expression to maintain a dynamic and responsive actin cytoskeleton.

Ectopic overexpression of either cytoskeletal Tm3 or Tm5NM1 in different mouse tissues had no significant impact on the expression of other cytoskeletal Tm isoforms. In an earlier in vitro study a similar outcome was observed (46). This is in direct contrast to observations that have been made previously for contractile proteins. Overexpression of β-Tm (15), a ventricular form of myosin light chain 2v (MLC2v) (14), MLC2f (16), essential light chain 1a (17), α-cardiac actin (18), and α-Tmslow carrying an amino acid substitution (M9AR) in skeletal muscle (21) in transgenic mice all elicit the down-regulation of a corresponding endogenous isoform. Moreover, down-

FIGURE 5. Cytoskeletal Tm4 is present within cardiac cells. Semithin (0.5–1.0 μm) longitudinal sections of adult NTG ventricle show the presence of Tm4 (WD4/9d antibody) near the Z-line (A) as shown by the presence of α-actinin (B). Merge of A (green) and B (red) shows Tm4 in the Z-LAC and in longitudinal filaments (arrow). Minimal background staining was observed when preimmune serum was used in place of the primary antibody (data not shown). Scale bars, 10 μm.
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The only known function of sarcomeric Tms is regulation of muscle contraction. In contrast, there is a much greater diversity of cytoskeletal Tm functions. The cytoskeletal Tm isoforms have been implicated in intracellular granule movement (47), vesicle budding within the Golgi complex (36, 48), cellular transformation (49), and cytokinesis (50), and they can differentially regulate the organization of actin filaments (6). Each of these functions is independently regulated; therefore, we propose that the lack of a feedback mechanism to maintain a pre-determined pool size of cytoskeletal Tm-containing filaments provides flexibility for the regulation of actin filaments with discrete functions. In this study, we demonstrate that ectopically expressed Tm5NM1 in primary embryonic fibroblasts is incorporated into the stress fiber compartment without spillover into the lamellipodia, mimicking the location of the endogenous Tm5NM1 isoform. We demonstrate that the ectopically expressed Tm5NM1 sorts to the Z-LAC within the soleus muscle as does the endogenous isoform. In addition, we showed previously that ectopic Tm5NM1 is restricted in location to the growth cone of neurons and results in increased growth cone size of primary embryonic cortical neurons (40). Hence, it is apparent that the cytoskeleton can accommodate altered levels of cytoskeletal Tm expression in restricted intracellular locations.

Not only are the cytoskeletal and sarcomeric Tm isoforms subject to differential regulation; they also localize to distinct subcellular compartments (for a review, see Ref. 51). This is particularly evident in muscle tissue, where sarcomeric Tms localize to the actin thin filaments of the sarcomere, and cytoskeletal Tms localize to cytoskeletal γ-actin-containing filaments in the Z-LAC that is distinct from the sarcomere (44). This compartmentalization is maintained even following transgenic overexpression. For example, ectopically expressed α-Tm localizes exclusively to the thin filaments of the sarcomere (21), whereas ectopically expressed cytoskeletal Tm3 localizes exclusively to the Z-LAC (44). The localization of Tm3 in muscle to cytoskeletal filaments is particularly striking given that this isoform is a high M, Tm, like muscle Tms and unlike Tm5, and is not usually expressed in muscle cells. This suggests the existence of a mechanism to regulate the subcellular distribution of sarcomeric versus cytoskeletal Tms. Indeed the targeting of isoforms to discrete subcellular locations happens despite potential deleterious effects; mutant α-Tm(low)(M9R) incorporates into the thin filaments within the sarcomeres and leads to nemaline myopathy (21), whereas ectopically expressed Tm3 localizes to the Z-LAC and elicits a muscular dystrophy phenotype (44). A possible reason for such spatial segregation is to confer distinct functional properties to Tm-containing microfilaments and ultimately to independently regulate these distinct pools of actin filaments.

The simplest interpretation of our data is shown in the model (Fig. 9). Existing data are consistent with the idea that a feedback regulatory mechanism controls the protein pool of sarcomeric Tms. Such a mechanism ensures that precise protein stoichiometry is maintained among the structural proteins in the sarcomere. In the case of the α-Tm knock-out mouse (27), translational control is suggested to play a major role in regulating Tm expression, whereas transgenic overexpression data

regulation of endogenous α-Tm protein also occurs when α-Tms containing mutations associated with familial hypertrophic cardiomyopathy (19, 20) are overexpressed. Conversely, knock-out of α-cardiac (22), α-skeletal (23), or smooth muscle actin (24) each correlate with an increase in the expression of an endogenous actin isoform. Hence, the synthesis of specific contractile proteins is regulated in part by feedback mechanisms that act to control the protein levels to ensure a strict stoichiometry is maintained.
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A possible explanation for the different regulatory mechanisms of sarcomeric and cytoskeletal Tms is that the structures they form ultimately dictate the accumulation of the respective Tms. Sarcomeric Tms accumulate in structures of fixed stoichiometry in striated muscle and do not appear to be able to displace cytoskeletal Tm4 from the cytoskeleton of cardiac striated muscle. Hence, the sarcomeric Tm pool size is fixed, and excess sarcomeric Tm is presumably subject to degradation. Conversely, neither Tm4 (Fig. 5), Tm5NM1 (44), nor Tm3 (44) can associate with sarcomeric actin in sarcomeres. Unlike the relationship between sarcomeric Tm and sarcomeric actin, the extra cytoskeletal Tm can accumulate and drive actin polymer formation.

Why do the sarcomeric and cytoskeletal Tms not compete for inclusion in the same structures? The different Tms have different affinities for actin (30, 56–59); however, unfortunately, most of these data were generated using only skeletal muscle actin. It is therefore unknown if differences in the actin isoforms, muscle versus cytoskeletal, could account for the lack of Tm isoform competition. Tm5NM1 is known to bind to muscle actin with similar affinity to that of muscle Tm (60, 61). The failure to detect Tm5NM1 in the sarcomere (44) (Fig. 6; this study), therefore, suggests that location and lack of competition are not simply a function of differential actin affinities. However, it is well established that Tm binding to actin is also influenced by simultaneous interaction with actin-binding proteins (59, 62, 63). It seems most likely that the collaboration of different Tms with spatially distinct populations of actin-binding proteins and actin isoforms is responsible for the preferred accumulation of Tm isoforms in separate actin filament populations (1, 51).

The differences in both the regulation and sorting of the Tm isoforms may potentially be useful in the derivation of new therapies for anti-cancer treatments. Actin has long been thought to be an attractive target for anti-cancer therapies; however, the ubiquitous expression of actin means that anti-actin compounds result in unacceptable associated toxicities in essential tissues, especially the heart and diaphragm (64). Inhibitors that target a specific population of actin filaments therefore provide an attractive approach for targeted therapy. Indeed, overexpression of nonmuscle Tm3 into CHO cells renders cells more sensitive to cytochalasin B treatment relative to other Tms (31). Our demonstration that cytoskeletal Tms are not subjected to feedback regulation together with their distinct subcellular compartmentalization (1) suggests that targeting cytoskeletal Tms may represent a novel and effective method to target specific actin cytoskeletal structures in the context of cancer chemotherapy (65).

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