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Tropomyosin isoforms and reagents

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Abbreviations: Tm, tropomyosin; GFP, green fluorescence protein; YFP, yellow fluorescence protein; LMW, low molecular weight; HMW, high molecular weight; RT-PCR, reverse transcription polymerase chain reaction; UTR, untranslated region; HA, hemagglutinin; kDa, kilodalton; aa, amino acid

Tropomyosins are rod-like dimers which form head-to-tail polymers along the length of actin filaments and regulate the access of actin binding proteins to the filaments.[1] The diversity of tropomyosin isoforms, over 40 in mammals, and their role in an increasing number of biological processes presents a challenge both to experienced researchers and those new to this field. The increased appreciation that the role of these isoforms expands beyond that of simply stabilizing actin filaments has lead to a surge of reagents and techniques to study their function and mechanisms of action. This report is designed to provide a basic guide to the genes and proteins and the availability of reagents which allow effective study of this family of proteins. We highlight the value of combining multiple techniques to better evaluate the function of different Tm isoforms and discuss the limitations of selected reagents. Brief background material is included to demystify some of the unfortunate complexity regarding this multi-gene family of proteins including the unconventional nomenclature of the isoforms and the evolutionary relationships of isoforms between species. Additionally, we present step-by-step detailed experimental protocols used in our laboratory to assist new comers to the field and experts alike.

Introduction

The actin microfilament system plays a critical role in a myriad of cellular functions including cell motility, cell division and cytokinesis, contractile force, cell signaling, transcription and intracellular transport. This wide range of biological functions is made possible by various actin binding proteins that associate with actin monomers or the filaments and modify any combination of the dynamics and mechanical and structural properties of cytoskeletal filaments. It is becoming increasingly clear that the actin filament is not a 'generic' passive player in this process but rather that the filaments themselves contain functional information which predisposes them to specific functional outcomes. One crucial component of filaments that confers a specific functional outcome is tropomyosin which forms a continuous polymer running along the major groove of most actin filaments.[1]

Tropomyosins (Tms) belong to a highly conserved and diverse family of actin associated proteins present in all animals from yeast to humans, with the possible exception of Dictyostelium, but absent in plants.[2] It is notable that in plants, however, there are more than the two cytoskeletal actins found in mammals and that the different actins provide functional discrimination between actin filaments.[3] Tm is characterized by its α-helical coiled-coil structure and dimers form an intermolecular head-to-tail complex resulting in a continuous elongated polymer that lies along the major groove of actin filaments.[4] The large number of documented Tm isoforms is derived from the use of alternative promoters and internal exon splicing of multiple genes.[5] A highly regulated repertoire of Tm isoforms is found in different cell types and expression of Tms is spatially and temporally regulated in a qualitative and quantitative manner (reviewed in refs. 6 and 7). Tms are recognized as actin filament stabilising proteins, regulating the dynamics and structural properties of the filaments by controlling the interaction of the filaments with actin binding proteins[1,8] and hence the term "gatekeeper" has been coined to describe the role of Tm.[9] For example, the high molecular weight (HMW) Tm isoforms are more efficient in protecting actin filaments from the severing activity of gelsolin than the lower molecular weight (LMW) isoforms whereas the HMW isoforms are better at annealing gelsolin severed filaments than LMW Tms.[10,11] The actin depolymerizing factor (ADF) and filamin compete with Tm for binding to actin filaments[12-15] in a Tm isoform specific manner.[16] Tms have also been shown to control the activity of myosin motors[17-20] and specify the intracellular sorting of myosin II in a Tm isoform specific manner.[16,21] Tm and caldesmon can also regulate myosin II activity in smooth muscle cells.[22] Finally, tropomodulins have been shown to have distinct affinities for different Tm isoforms.[23-25] Together with the findings that Tm isoforms sort to different subcellular compartments, these observations strongly suggest that this multitude of isoforms is required for the

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Table 1. Ensemble identification numbers of the Tm genes from different species, chromosome location and adjacent genes

Species	Gene	Ensemble Accession No.	Chromosome	Adjacent Loci
Human (*Homo sapians*)	TPM1 (αTm)	ENSG00000140416	Ch 15	Talin2, Rab8b, CA12
	TPM2 (βTm)	ENSG00000198467	Ch 9	Talin1, RUSC2, CREB3, GBA2
	TPM3 (γTm)	ENSG00000143549	Ch 1	HAX1, SNORA58.2, Rab13, RUSC1
	TPM4 (δTm)	ENSG00000167460	Ch 19	Rab8a
Mouse (mus musculus)	TPM1 (αTm)	ENSMUSG00000032366	Ch 9	Talin2, Rab8b, CA12
	TPM2 (βTm)	ENSMUSG00000028464	Ch 4	Talin1, RUSC2, CREB3, GBA2
	TPM3 (γTm)	ENSMUSG00000027940	Ch 3	HAX1, SNORA58.2, Rab13, RUSC1
	TPM4 (δTm)	ENSMUSG00000031799	Ch 8	Rab8a
Rat (*Rattus norviegicus*)	TPM1 (αTm)	ENSRNOG00000018184	Ch 1	Talin2, Rab8b, CA12
	TPM2 (βTm)	ENSRNOG00000016731	Ch 5	Talin1, RUSC2, CREB3, GBA2
	TPM3 (γTm)	ENSRNOG00000017441	Ch 2	Rab13, RUSC1
	TPM4 (δTm)	ENSRNOG00000015496	Ch 16	Rab8a
Chicken (*Gallus gallus*)	TPM1 (αTm)	ENSGALG00000003521	Ch 10	Talin2, Rab8b, CA12
	TPM2 (βTm)	ENSGALG00000002563	Ch Z	Talin1, RUSC2, CREB3, GBA2
	TPM3 (γTm)	ENSGALG00000013537	Ch 25	SNORA58, RUSC1
	TPM4 (δTm)	-	Ch 28	Rab8a
Western Clawed frog (*Xenopus tropicalis*)	TPM1 (αTm)	ENSXETG00000009718	-	-
	TPM2 (βTm)	ENSXETG00000009615	-	-
	TPM3 (γTm)	ENSXETG00000020732	-	-
	TPM4 (δTm)	ENSXETG00000008736	-	-
Zebrafish (*Danio rerio*)	TPM1 (αTm)	ENSDARG00000087402	Ch 25	Talin2
	TPMa (αTm)	ENSDARG00000033683	Ch 7	Talin2 (homologue) fbxo22
	TPM2 (βTm)	ENSDARG00000068385	Ch 10	Talin1
	TPM3 (γTm)	ENSDARG00000005162	Ch 19	MSMP, tomm40l, arhgef1
	TPM4-1 (δTm)	ENSDARG00000019128	Ch 2	Rab8a
	TPM4-2 (δTm)	ENSDART00000142717	Ch 22	ANKMY1

proper spatial and temporal regulation of cytoskeletal function.[1]

In this report we document the many tools currently available to study the biological function of this family of proteins including Tm isoform specific antibodies, mammalian expression constructs and mouse models. The surprisingly high degree of amino acid conservation that exists in organisms as divergent as fish and humans indicates that such tools as the Tm antibodies can be utilized in multiple model systems. We envisage that this report will serve as a self-help guide to the study of the role of Tm isoforms in the cytoskeleton.

Results and Discussion

The generation and evolution of Tm isoforms. The number of Tm genes varies from one and two in the fungi *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively, four in mammals and six in zebra fish (*Danio rerio*).[26,27] **Table 1** lists the genes identified in 6 commonly studied animal model systems together with the chromosome location and the genes found adjacent to the Tm loci. Interestingly, the Tm loci are found adjacent to a few common genes including talin and Rab8a. For the most part the focus of this report will be on mammalian Tm genes and isoforms. A schematic representation of the intron-exon organization of the four mammalian genes is shown in **Figure 1**. The overall organization of exons and splicing patterns in the mammalian genes is conserved between mammals, avian, amphibians and fish. The genes in simpler species follow similar principles but differ substantially in the details of alternative exons and their evolutionary relationship to the specific genes in vertebrates.[27] The human Tm genes have been named TPM1, TPM2, TPM3 and TPM4, however, due to the lack of a unified nomenclature for the gene names or the isoforms, alternative names documented in the literature are listed in **Table 2**.

Figures 2–5 show, for each gene, the amino acid sequences of each of the exons compared between mammals, birds, amphibians and fish. The consensus sequence across species is shown below each exon. The most striking feature of this comparison is the extraordinary conservation of primary structure across over 500 million years of evolution. This emphasizes that these proteins are under great selective pressure to maintain a precise primary sequence. Selection is unlikely to reflect the structural demands of maintaining the coiled coil because there are few absolute primary sequence

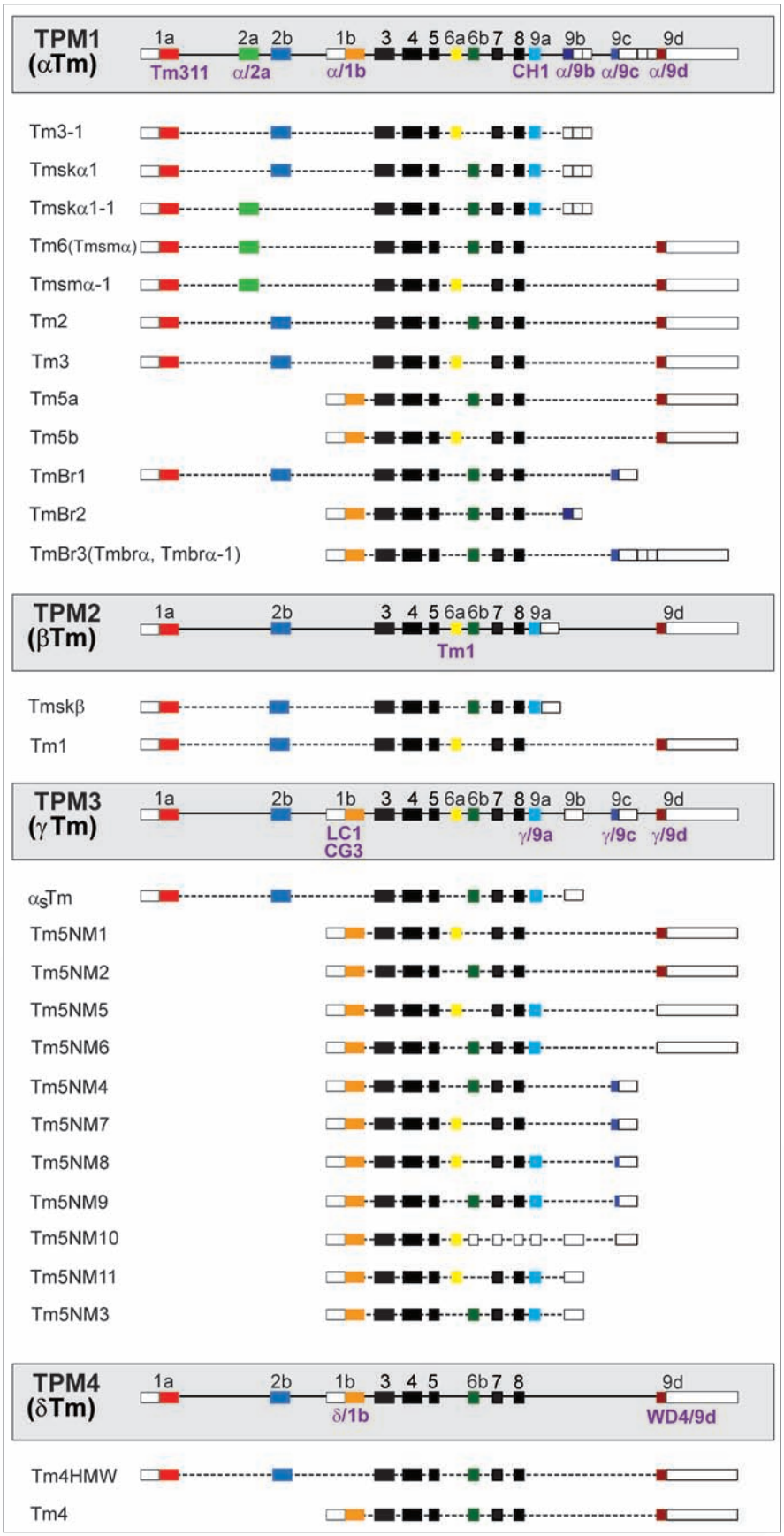

Figure 1. Schematic representation of the organization of the mammalian Tm genes. Colored boxes represent the protein coding exons numbered 1 to 9 and lines the introns. Unshaded boxes signify untranslated sequences and black boxes are exons common to all the genes which share a high degree of homology. The various isoforms generated via alternative exon splicing are listed under each gene and only the major products verified by northern or protein gel blots are shown. The name of the different Tm antibodies (denoted in purple) is below the exon containing the epitope.

requirements necessary to generate a coiled coil.[28] Rather, the selection is more likely to reflect isoform specific functional demands[1] possibly underpinned by subtle structural features as described recently in reference 29 and/or positioning in the major groove of the actin filament.[30,31]

Sequence diversity between the isoforms results from both sequence divergence between alternative exons in the same gene and between the same exons in different genes (**Fig. 6**). Comparison of the amino acid sequences of each of the 4 human genes, exon by exon, shows that there is a high degree of sequence similarity between the genes although this varies between exons (**Fig. 6A**). Some exons, like 1a, 2b, 3, 4, 5, 6b, 7, 8 and 9a show a high degree of similarity between the genes whereas exons 1b, 6a, 9c and 9d show substantial differences which would be expected to contribute to isoform divergence (**Fig. 6A**). The greatest source of primary sequence divergence comes from alternative promoter use and alternative splicing. There are two types of N-termini which result from the use of different promoters (**Fig. 1**). Exon 1a plus either exon 2a or 2b gives rise to the N-terminus of high molecular weight tropomyosins (approximately 284 aa). The alternative N-terminus derives from the use of exon 1b and gives rise to the shorter LMW isoforms (approximately 248 aa). In addition to the obvious length difference between the choices of N-terminus, there is also considerable sequence divergence (**Fig. 6B**) between these alternative N-termini. Thus the similarity between genes for the same exons is much greater than it is between alternative exons in the same gene. This trend is also seen for exons 6a vs. 6b and also for the C-terminal exons

Table 2. Alternative names for mammalian Tms

Gene Name	Gene alternative names	Tm isoforms	Isoform alternative names	NCBI accession numbers for the human isoforms
TPM1	αTm	Tm3-1	-	DNA-NM_000366, Protein-NP_000357
		αf-Tm	$αTm_{fast}$, $Tm_{skα}$, hTmskα1	DNA-NM_001018005, Protein-NP_001018005
		hTmskα1-1	TPM1κ	DNA-AY640414, Protein-AAT68294
		hTmsmα	Tmsmα, Tm6	DNA-NM_001018007, Protein-NP_001018007
		hTmsmα-1	-	DNA-NM_001018020, Protein-NP_001018020
		Tm2	-	DNA-NM_001018004, Protein- NP_001018004
		Tm3	-	DNA-NM_001018006, Protein-NP_001018006
		Tm5a	-	DNA-CH471082 AADB02000000, Protein-EAW77629
		Tm5b	-	DNA-CH471082 AADB02000000, Protein-EAW77633
		TmBr1	-	DNA-CH471082 AADB02000000, Protein-EAW77630
		TmBr2	-	
		TmBr3	hTmbrα-1, hTmbrα	DNA-AB209041, Protein-BAD92278
TPM2	βTm	β-Tm	Tmskβ, hTmskβ	DNA-NM_003289, Protein-NP_003280
		Tm1	Smooth muscle Tm1-Fibroblast	DNA-NM_213674, DNA-NM_213674
		hTm1-1	-	DNA-CR590682, Protein-None
TPM3	γTm, $hTm_{30\ nm}$, hTm_{nm}, Tm-5	$α_s$-Tm	$αTm_{slow}$, $α_s$Tm, hTmskα2	DNA-NM_152263, Protein-NP_689476
		Tm5NM1	hTm5, Tm5, Tm30 , fibroblast Tm30 nm	DNA-NM_153649, Protein-NP_705935
		Tm5NM2	-	DNA-NM_001043351, Protein-NP_001036816
		Tm5NM6	-	DNA-CH471121, Protein-EAW53237
		Tm5NM7	hTm5-1, TC22	DNA-NM_001043352, DNA-NM_001043352
		Tm5NM4	hTmbrγ	DNA-NM_001043353, Protein-NP_001036818
		Tm5NM8	-	
		Tm5NM9	-	
		Tm5NM10	-	
		Tm5NM11,5	hTm5-2	DNA-AL590431, Protein-CAH71266
		Tm5NM3	-	
TPM4	δTm, hTm30pl, hTmpl, Tm-4	hTm4HMW	-	DNA-AK023385, Protein-BAB14554
		Tm4	-	DNA-NM_003290, Protein-NP_003281

9a, 9b, 9c and 9d. In both cases, it is almost impossible to detect any homology between the alternative exons in the same gene (**Fig. 6B**). This divergence has provided an effective strategy for the generation of antibodies which recognize specific subsets of isoforms (see below).

RT-PCR analysis has identified over 40 Tm isoforms that are coded by the mammalian genes.[5,32,33] Evidence for the presence of transcripts detected at the level of northern blot analysis or most preferable at the protein level only really exists for half of these isoforms and the majority are listed in **Table 2**. Tm isoforms found in association with the contractile apparatus of skeletal, cardiac and smooth muscle cells are referred to as muscle Tms whereas those associated with the cytoskeleton of all cells are referred to as the cytoskeletal isoforms (often referred to, incorrectly, as non-muscle isoforms in the literature).

TPM1 and TPM2 are also referred to as the αTm and βTm gene respectively[34] (**Fig. 1**). The TPM1 gene has been found by RT-PCR to code for 20 isoforms in rat adult tissues and cultured cells.[32] Included among the encoded isoforms is the αTm expressed in striated muscle, the recently discovered TPM1κ in human hearts,[35] the isoforms preferentially enriched in the central nervous system, TmBr1, TmBr2 and TmBr3 and Tm2, Tm3, Tm5a and Tm5b. The TPM2 gene codes for the skeletal muscle βTm isoform and, thus far, only one cytoskeletal isoform, Tm1, has been detected. Tm1 has been found to be significantly downregulated in transformed cells and restoring Tm1 expression has been shown to reverse transformation in ras-transformed cells.[36] The TPM3 gene codes for the slow-twitch skeletal muscle isoform ($α_s$Tm) and at least 9 LMW cytoskeletal isoforms referred to as Tm5NM1 to Tm5NM11.[33,37-39] Isoforms NM3 and NM6 are identical at the protein level but have unique 3'UTRs, the same is for isoforms NM5 and NM11 (**Fig. 1**). The TPM4 gene[40,41] codes for the cytoskeletal Tm4 isoform and a HMW Tm4 found in many species but not rodents.[42]

Post translational modifications. To date post translational modifications of Tms are largely an unexplored area of investigation. The most reported forms of post translational modifications in the literature are NH_2-terminal acetylation and phosphorylation although evidence of S-nitrosylation and disulfide-crosslinked Tm has been documented.

Acetylation has been shown to significantly enhanced Tm affinity for actin. Unacetylated mammalian skeletal Tm is unable to bind actin in the absence of troponin[43,44] and unacetylated α-smooth muscle Tm has much weaker affinity for actin than the acetylated form.[45] In addition, acetylation has been shown to increase the end-to-end interactions between Tm dimers[9,44,46] and in *Schizosaccharomyces pombe* to significantly enhance the ability of Tm to regulate

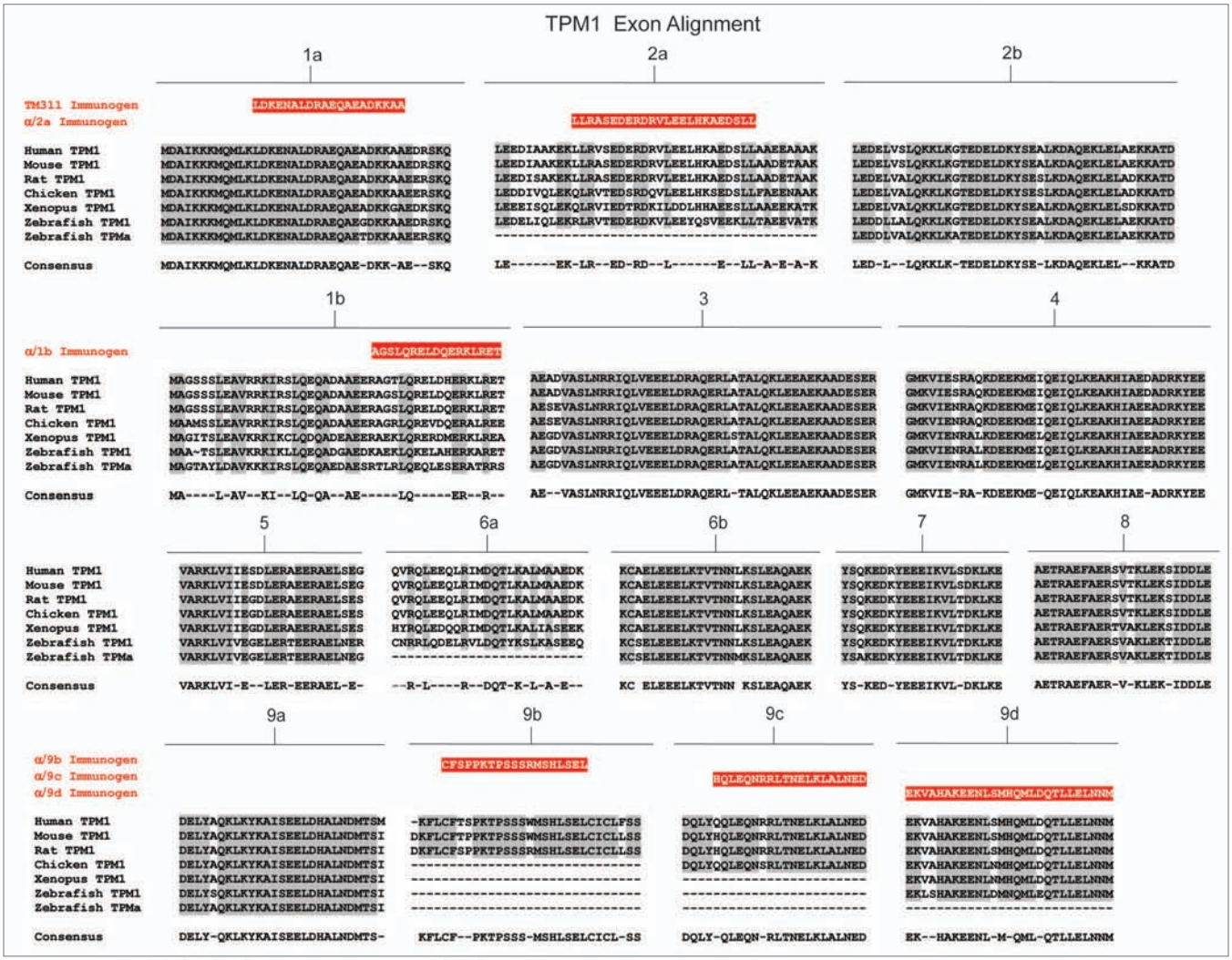

Figure 2. Amino acid sequence comparison of the TPM1 gene from six animal species. Amino acid alignment of all exons contained within the TPM1 gene for selected members of chordata. Species included are human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norviegicus*) representing mammalians, chicken (*Gallus gallus*) representing birds, western clawed frog (*Xenopus tropicalis*) representing amphibians and zebrafish (*Danio rerio*) representing fish. Shaded areas and consensus sequence indicate regions of sequence conservation while unshaded regions represent areas of sequence divergence between the aligned species. Dashed lines within alignments imply that either the particular exon is not found within that species or that the exon in question has not been identified due to minimal genomic sequence information and lack of evidence from cDNA library sequences. Zebrafish contains two copies of the TPM1 gene named TPM1 and TPMa which are the result of a whole genome duplication event. Antibody immunogens and/or epitopes for antibodies TM311, α/2a, α/1b, α/9b, α/9c and α/9d are presented as white text highlighted in red and are aligned with the region of the exon from which they were derived.

myosin activity.[47] At least in yeast, distinct spatial segregation of acetylated and non-acetylated Tm-containing actin filaments has been observed suggesting the possibility that different forms of Tm can regulate specific myosins.[17]

Phosphorylation of Tm has been found in both skeletal and cardiac muscle of many different species.[48-54] The phosphorylation of Tm has been shown to enhance the ability of Tm to form head-to-tail interactions and promote activation of myosin Mg^{2+} ATPase.[55,56] Phosphorylation of cytoskeletal Tms has also been documented and postulated to be required for the remodelling of the actin cytoskeleton.[57,58]

Finally, endothelial cells exposed to shear flow were shown to have more than 12 proteins that had significantly increased S-nitrosylation among them Tm at Cys 170 located in the hydrophobic motif.[59] The authors suggest that such modification may allow for the remodelling together with maintaining the integrity of the endothelial cells under flow conditions.

Evaluating the specificity of Tm antibodies. Many of the Tm antibodies commercially available are sold with minimal information regarding the antigen used to raise the antibody and hence the isoform specificity. This greatly limits their use and in some cases leads to confusion in the literature. We previously described the characterization of 10 Tm antibodies.[60] Here, we report an additional 9 Tm antibodies. **Table 3** is a comprehensive list of all the Tm antibodies that we have extensively characterized together with

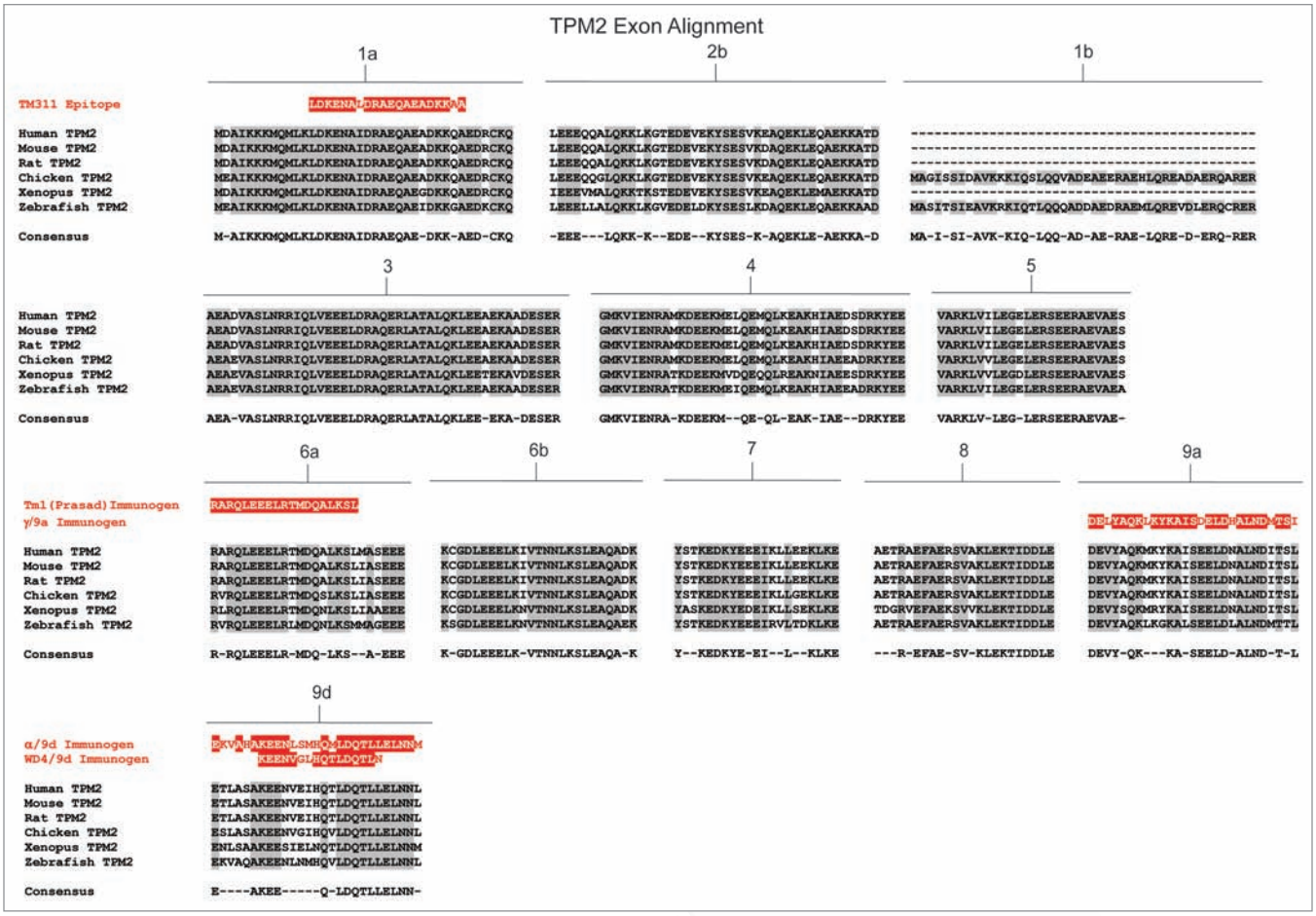

Figure 3. Amino acid sequence comparison of the TPM2 gene from six animal species. Amino acid alignment of all exons contained within the TPM2 gene for selected members of chordata. Species included are human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norviegicus*) representing mammalians, chicken (*Gallus gallus*) representing birds, western clawed frog (*Xenopus tropicalis*) representing amphibians and zebrafish (*Danio rerio*) representing fish. Shaded areas and consensus sequence indicate regions of sequence conservation while unshaded regions represent areas of sequence divergence between the aligned species. Dashed lines within alignments imply that either the particular exon is not found within that species or that the exon in question has not been identified due to minimal genomic sequence information and lack of evidence from cDNA library sequences. Antibody epitopes/immunogens for antibodies TM311, Tm1 (Prasad), δ/9d, γ/9d and α/9d are presented as white text highlighted in red. The Tm1 (Prasad) immunogen is designed to the TPM2 6a exon, whereas the other immunogens are designed to exons in other Tm genes. Shaded and unshaded areas within the immunogen sequence reflect sequence similarity and divergence with the corresponding sequence in the TPM2 gene.

the isoform specificity, published references and commercial availability. The majority of the antibodies were generated using peptides corresponding to part or all of a specific exon. There are exceptions where the initial antibodies were made by using whole tissue tropomyosin, for example chicken gizzard, and the epitopes are yet to be identified. **Figure 1** shows the name of the antibody below the exon encoding the epitope and the peptide used as immunogen for these antibodies is shown in **Figures 2–5**. Comparison of the peptide sequence containing the epitope cross species indicates the likelihood that many of these antibodies will be reactive across many model systems (**Figs. 2–5**).

The initial evaluation on the specificity of the antibodies was performed by protein gel blot analysis on panels of recombinant Tm proteins. The corresponding cDNAs of rat (Tm1, 2, 3, 4, 5a, 5b, Br1, Br2, Br3 and Tm5NM1) and human (Tm1, 2, 3, 4, NM1, NM2, NM4, NM5, NM6, NM7) isoforms were cloned into the pPROEX HT prokaryotic or pET expression system and induction of protein was performed as described in Materials and Methods.

A total of 13 antibodies have been generated that detect Tm isoforms from the TPM1 and 2 genes with three of these Tm antibodies having been generated as both polyclonals and monoclonals. The epitope for the commercially available TM311 antibody has been mapped to exon 1a of the TPM1 gene and thus it detects the striated muscle isoform plus Tms 2, 3, 6, Br1 and Tm1 plus the striated muscle isoform from the TPM2 gene (**Figs. 1 and 7A**). Isoforms containing exon 1b (Tm5a, 5b, Br2 and Br3) are detected with the α/1b antibody (**Fig. 7B**). Antibodies with epitopes directed to the C-terminal exon of the αTm gene include the α/9b antibody specific for TmBr2 (**Fig. 7C**) and the α/9c (clone #554) which preferentially detects TmBr1 and TmBr3 (**Fig. 7D**). It is notable that α/9c does not see Tm5NM4 which contains exon 9c from the TPM3 gene. The α/9d antibody raised against a peptide directed to the entire exon 9d

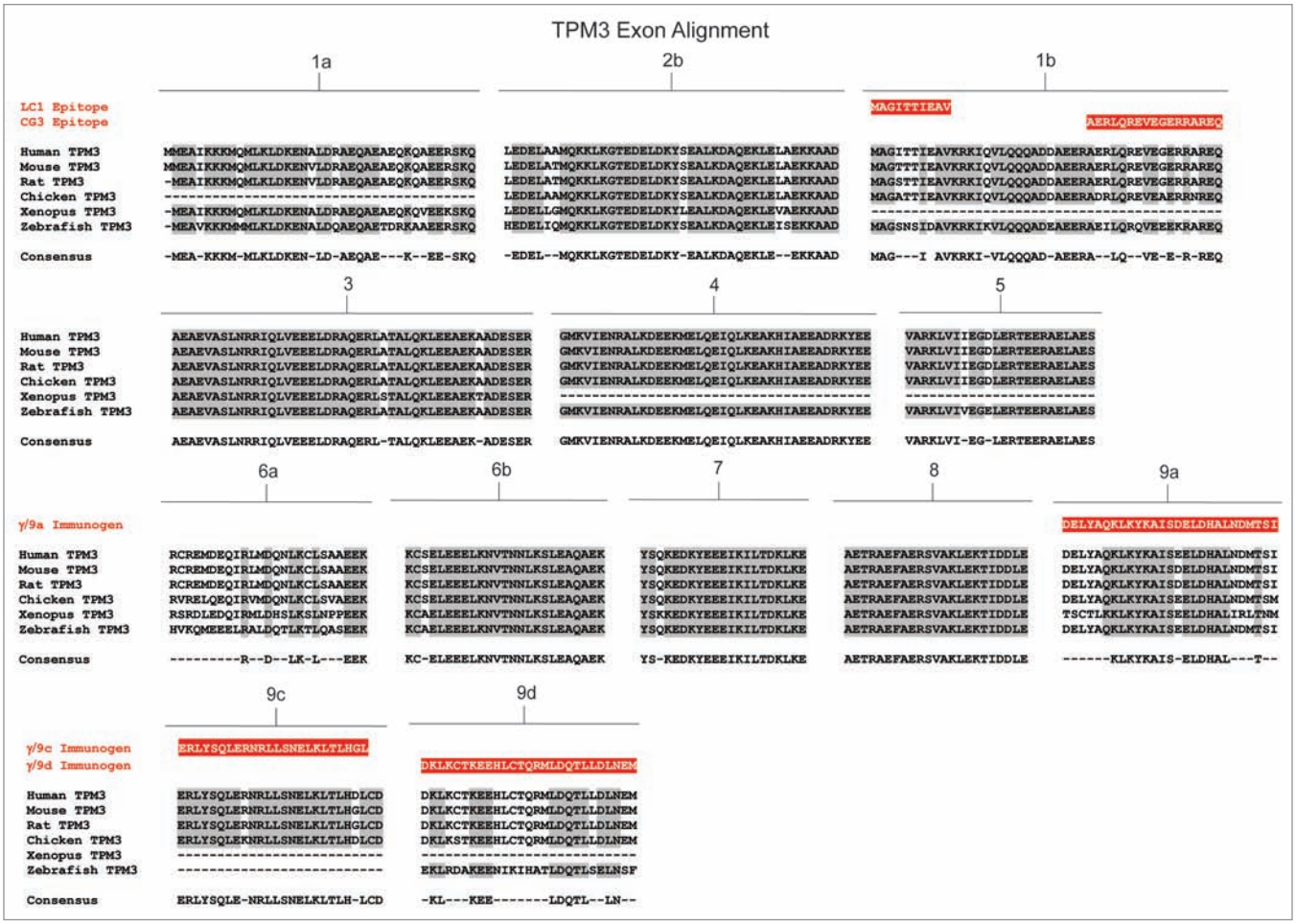

Figure 4. Amino acid sequence comparison of the TPM3 gene from six animal species. Amino acid alignment of all exons contained within the TPM3 gene for selected members of chordata. Species included are human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norviegicus*) representing mammalians, chicken (*Gallus gallus*) representing birds, western clawed frog (*Xenopus tropicalis*) representing amphibians and zebrafish (*Danio rerio*) representing fish. Shaded areas and consensus sequence indicate regions of sequence conservation while unshaded regions represent areas of sequence divergence between the aligned species. Dashed lines within alignments imply that either the particular exon is not found within that species or that the exon in question has not been identified due to minimal genomic sequence information and lack of evidence from cDNA library sequences. Antibody epitopes and immunogens for antibodies LC1, CG3, γ/9a, γ/9c and γ/9d are presented as white text highlighted in red and are aligned with the region of the exon from which they were derived.

from the TPM1 gene has a high degree of specificity for Tm1, 2, 3, 5a and 5b and, we predict, Tm6 (**Fig. 1**). Although exons 9d from the TPM1, 3 and 4 genes have a high degree of similarity, the α/9d antibody exhibits no cross reactivity with either Tm4 from the TPM4 gene nor Tm5NM1 and Tm5NM2 from the TPM3 gene (**Fig. 7E**). The CG1 and CGβ6 antibodies were originally raised against chicken gizzard Tm.[61] CG1 has strong reactivity to both rat and human Tm1 and also detects Tm4 (**Fig. 7F**). The epitope for the CGβ6 antibody has been mapped to the C-terminal end of human Tm2 and Tm3 [62] and as expected it detects both human and mouse Tm2 and Tm3 and also shows weak reactivity with Tm5a (**Fig. 7G**).

Peptide sequences corresponding to the alternatively spliced C-terminal exons of the TPM3 gene (exons 9a, 9c and 9d) were used to generate the γ/9a, γ/9c and γ/9d antibodies (**Table 3**). The characterization of these antibodies raised in sheep has been previously documented in reference 60. Here we report on the complementary antibodies generated using the identical peptide sequences but raised as mouse monoclonals. These three antibodies show the predicted isoform specificity (**Fig. 8A–C**). The γ/9d antibody does exhibit minor cross reactivity with Tm1, 2, 3, 5a and 5b (**Fig. 8C**). The LC1 antibody is shown to have a high degree of specificity for the human isoforms Tm5NM1, NM2, NM4 and 7 with no cross reactivity to the mouse Tm5NM1 (**Fig. 8D**). Although the epitope for this antibody is yet to be mapped our data and that reported by Sung et al. strongly indicate that it resides in the first few N-terminal end amino acids of the human exon 1b from the TPM3 gene. Amino acid sequence alignment demonstrates that the only amino acid difference between the mouse/rat and human sequences is the fourth amino acid, isoleucine in human and serine in mouse and rat (**Fig. 4**). We expect that this residue must be in the LC1 epitope. The CG3 antibody with its epitope mapped to exon

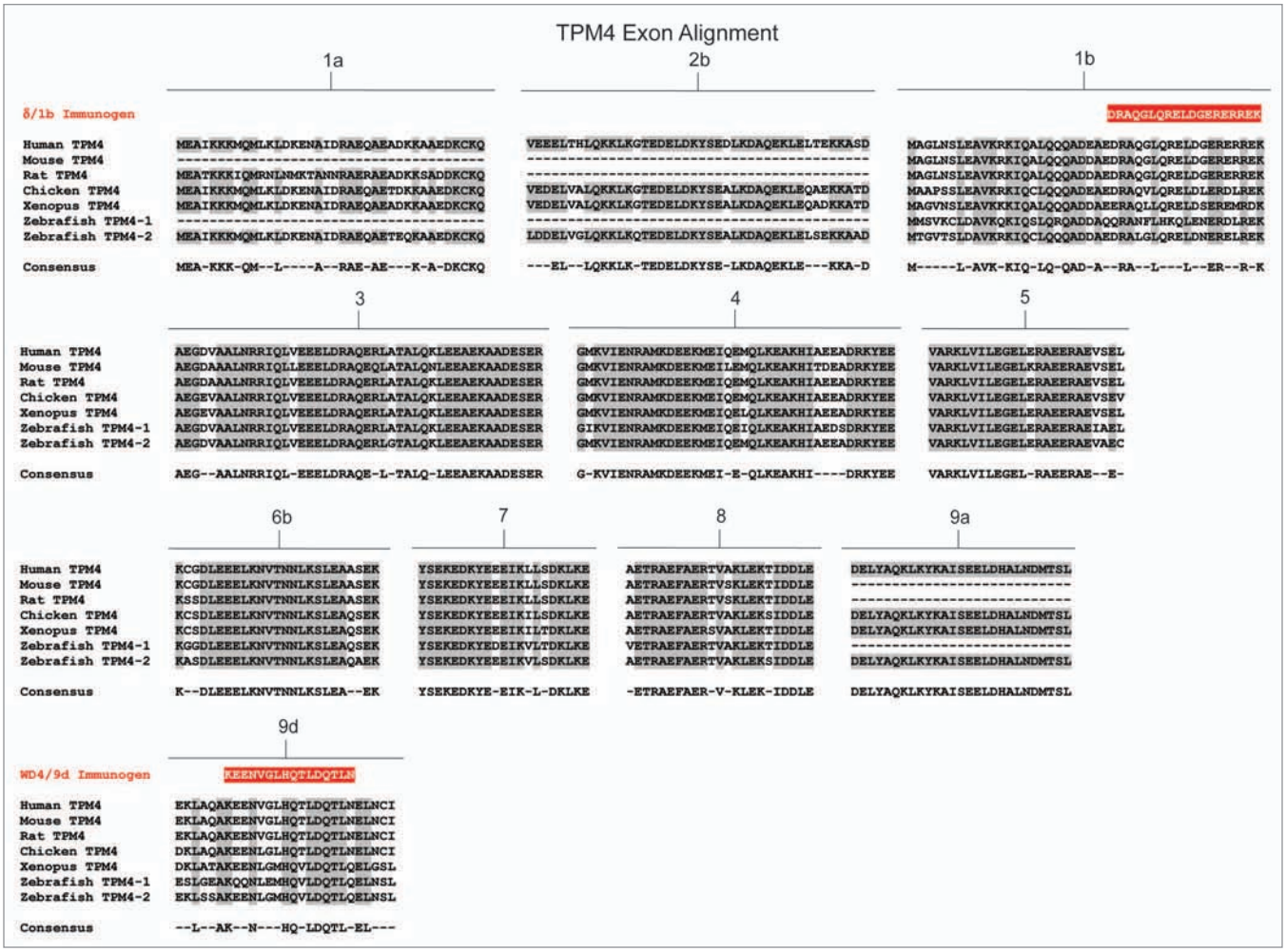

Figure 5. Amino acid sequence comparison of the TPM4 gene from six animal species. Amino acid alignment of all exons contained within the TPM4 gene for selected members of chordata. Species included are human (*Homo sapiens*), mouse (*Mus musculus*) and rat (*Rattus norviegicus*) representing mammalians, chicken (*Gallus gallus*) representing birds, western clawed frog (*Xenopus tropicalis*) representing amphibians and zebrafish (*Danio rerio*) representing fish. Shaded areas and consensus sequence indicate regions of sequence conservation while unshaded regions represent areas of sequence divergence between the aligned species. Dashed lines within alignments imply that either the particular exon is not found within that species or that the exon in question has not been identified due to minimal genomic sequence information and lack of evidence from cDNA library sequences. Zebrafish contains two copies of the TPM4 gene, named TPM4-1 and TPM4-2 which are the result of a whole genome duplication event. Antibody immunogens for antibodies δ/1b and WD4/9d are presented as white text highlighted in red and are aligned with the region of the exon from which they were derived. The antibody immunogen for γ/9a (TPM3 gene) is also shown.

1b amino acids 29–44 [64] detects all the cytoskeletal products from this gene both human and mouse (**Fig. 8E**). It has also been found to detect the striated muscle isoform from this gene via cross reaction with the TPM3 exons 1a or 2b.[65]

The antibodies known to specifically detect the cytoskeletal Tm4 isoform from the TPM4 gene are δ/1b, WD4/9d and LC24. The δ/1b antibody was raised against the last 19 amino acids of exon 1b from the TPM4 gene. This antibody is shown to detect both rat and human Tm4 and shows no cross reactivity to other exon 1b containing isoforms from the other Tm genes (Tm5a, 5b, Br2, Br3, NM1, NM2, NM4, NM7) (**Fig. 9A**). It does however, show weak reactivity with human Tm1. The WD4/9d generated in rabbit using a partial amino acid sequence from exon 9d of the TPM4 gene has been previously characterized.[60] It detects Tm4 but does cross react with Tm1 from the βTm gene (on a longer exposure, data not shown), and also Tm5NM1 and Tm5NM2 from the γTm gene (**Fig. 9B**). The LC24 was generated against bacterially produced human Tm4 with only the C-terminal end used.[62] This antibody detects both rat and human Tm4 but also cross reacts with Tm1 from the TPM2 gene (**Fig. 9C**).

The use of Tm antibodies to identify the Tm isoform expression profiles in cell lines and mouse tissues. The selective repertoire of Tm isoform expression, including the number and type of isoforms expressed in different cell types is strictly regulated by as yet not fully understood mechanisms. Tm isoform expression profiles in different cell types have previously been reviewed in reference 6. Here we report the use of the Tm antibodies described above to profile isoform expression in three different cell types

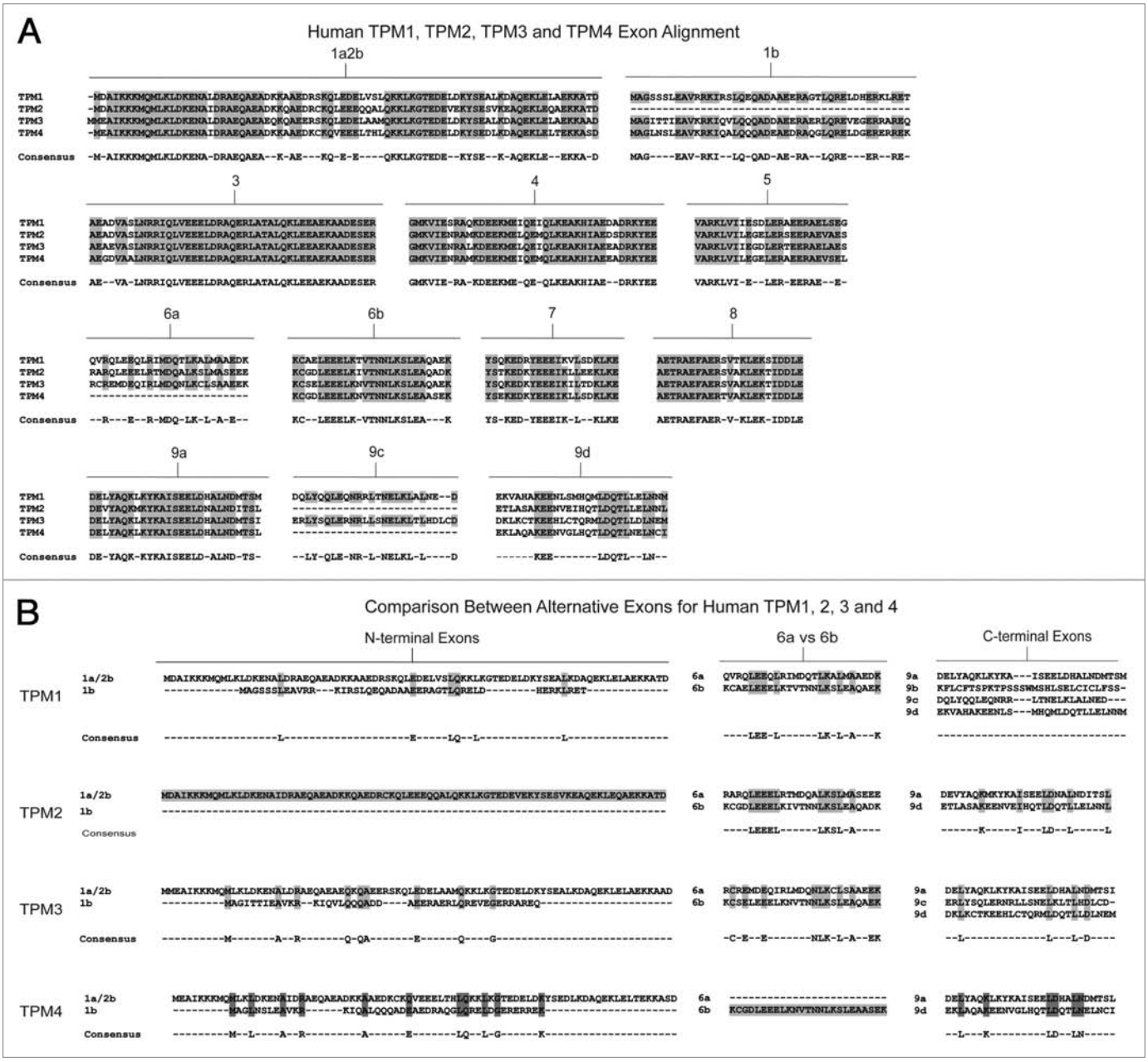

Figure 6. Exon alignment of the human Tm genes. A comparison highlighting the differences in amino acid composition between an alignment of corresponding exons from human TPM1, TPM2, TPM3 and TPM4 genes and an alignment of the exons responsible for generating isoforms; exons 1a/2b and 1b (N-terminus), 6a and 6b, and exons 9a, 9b, 9c and 9d (C-terminus) within each of the 4 Tm genes. (A) An alignment of human Tm genes TPM1, TPM2, TPM3 and TPM4 showing the conservation of amino acid sequence between the four genes. Shaded residues and consensus sequence indicate regions of conservation within the genes whilst unshaded regions indicate areas of sequence divergence. (B) An alignment of exons responsible for generating diversity showing amino acid sequence conservation for alternatively spliced exons from the N-terminus, middle and C-terminus within each of the four genes. Shaded and unshaded areas are as indicated above.

from three different mammalian species, human, mouse and rat and further evaluate the specificity of these tools. On a SDS PAGE gel the HMW Tm isoforms have an apparent molecular weight of 34 to 40 kDa and the LMW isoforms of 28 to 33 kDa. Migration of Tms in SDS gels is not predictable based on MW and in our experience, optimal resolution of Tm isoforms is best achieved on SDS-PAGE gels with a low concentration of bisacrylamide (12.5% acrylamide and 0.1% bisacrylamide).[66] The antibodies known to detect isoforms from the TPM1 and TPM2 genes including TM311, α/9d, 1 and CGβ6 all perform well on total protein lysates isolated from cell lines (**Fig. 10**). The initial observation made on the protein gel blots is the differences in the profiles of isoforms and the quantitative amounts expressed by the three cell lines. Both the human and mouse cell lines express Tm1 as detected with the TM311, α/9d and CG1 antibody (**Fig. 10A, C**

Table 3. Summary of Tm antibodies, exon and isoform specificity

Antibody name TPM1, 2 gene antibodies	Peptide sequence/Immunogen	Exon specificity	Tm isoform recognition	Reference	Species	Commercial availability
TM311	LDKENALDRAEQAEADKKAA	aa14-32 exon 1a	Tm6, 1, 2, 3, Br1 α,β,γ muscle Tm	[98]	mouse monoclonal	Sigma Aldrich
α/1b	CAGSLQRELDQERKLRET	exon1b	Tm5a, 5b, Br2, Br3	this paper	sheep polyclonal	
α/2a	CLLRAESEDERDRVLEELHKAEDSLL	exon 2a	sm mus Tm (Tm6)	[99]	sheep	Millipore AB5437
α/2a	CLLRAESEDERDRVLEELHKAEDSLL	exon 2a	sm mus Tm (Tm6)	this paper	mouse monoclonal	
α/9b	CFSPPKTPSSSRMSHLSEL	exon 9b	TmBr2	this paper	mouse monoclonal	
WSα/9c	HQLEQNRRLTNELKLALNED	exon 9c	TmBr-1, TmBr-3	[60, 100]	rabbit	Millipore AB5439
α/9c	HQLEQNRRLTNELKLALNED	exon 9c	TmBr-1, TmBr-3	[68]	mouse monoclonal clone #554	
α/9d	EKVAHAKEENLSMHQMLDQTLLELNNM	entire exon 9d	Tm6, 1, 2, 3, 5a, 5b	[60]	sheep	Millipore AB5441
α/9d	EKVAHAKEENLSMHQMLDQTLLELNNM	entire exon 9d	Tm6, 1, 2, 3, 5a, 5b	this paper	mouse monoclonal (IgG2bk)clone15D12.2	
CG1	Chicken gizzard Tm	Unknown	Tm1	[61, 101]	mouse monoclonal (IgG1)	
CGβ6	Chicken gizzard Tm	COOH terminal end of hTm2, htm3	Tm2, 3	[61, 62]	mouse monoclonal (IgM)	
Sarcomeric Tm (Sigma CH1)	Unknown.	exon α9a	Sarcomeric Tms: αfast, β, αslow (poor affinity for αslow)	[60, 61]	mouse monoclonal (IgG)	Sigma Aldrich
Tm1(Prasad)	SRARQLEEELRTMDQALKSL (187-206aa)	exon β6a	Tm1	[102]	rabbit polyclonal	
Antibody name TPM3 gene antibodies	**Peptide sequence/Immunogen**	**Exon specificity**	**Tm isoform recognition**	**Reference**	**Species**	**Commercial availability**
γ/9a	CDELYAQKLKYKAISDELDHALNDMTSI	exon 9a	Tm5NM6/3, 5/11, 8, 9, α and β Tms	[68]	sheep polyclonal	Millipore AB5443
γ/9a	CDELYAQKLKYKAISDELDHALNDMTSI	exon 9a	Tm5NM6/3, 5/11, 8, 9, α and β Tms	this paper	mouse monoclonal	
γ/9c	CERLYSQLERNRLLSNELKLTLHGL	exon 9c	Tm5NM4, 7	[68]	sheep polyclonal	Millipore AB5445
γ/9c	CERLYSQLERNRLLSNELKLTLHGL	exon 9c	Tm5NM4, 7	this paper	mouse polyclonal (IgG1k) clone 3F8.2	
γ/9d	DKLKCTKEEH LCTQRMLDQTLLDLNEM	exon 9d	Tm5NM1, 2	[60]	sheep polyclonal	Millipore AB5447
γ/9d	DKLKCTKEEH LCTQRMLDQTLLDLNEM	exon 9d	Tm5NM1, 2	this paper	mouse monoclonal (IgG2bk)clone2G10.2	
LC1	First 10 amino acids of Human exon γ1b	exon 1b	human Tm5NM1, 2	[63]	mouse monoclonal (IgG1)	
CG3	AERLQREVEGERRAREQ	aa29-44 exon1b	All nonmuscle products from the γTm gene	[61, 64]	mouse monoclonal (IgM)	
Antibody name TPM4 gene antibodies						
δ/1b	DRAQGLQRELDGERERREK	exon 1b	Tm4	this paper	mouse monoclonal	
WD4/9d	KEENVGLHQTLDQTLNEL	exon 9d	Tm4	[60, 103]	rabbit polyclonal	Millipore AB5449
LC24	COOH terminal half of hTm4	exon 9d	Human, rat Tm4	[61, 62]	mouse monoclonal (IgG1)	

and D). The rat B35 cells show no evidence of Tm1 or Tm3 expression whereas Tm2 and Tm3 are detected in both the human and mouse cells and only Tm2 in the rat B35 cells (**Fig. 10A and E**). The α/1b antibody detects very low levels of Tm5a and Tm5b but there is also reactivity with unknown bands (**Fig. 10B**). Antibodies directed to Tm isoforms enriched in brain tissue (TmBr1, Br2 and Br3) and smooth muscle (Tm6) were as predicted absent from these cell lines (data not shown).

All the TPM3 antibodies tested on the cell lines were found to detect the expected cytoskeletal LMW isoforms coded by the gene (**Fig. 11**). The abundance of isoforms detected with the γ/9a antibody was found to be low as a very long exposure time was used to generate the blot shown in **Figure 11A**. Some additional unknown bands were also detected. The γ/9d antibody detects the predicted 30 kDa corresponding to Tm5NM1/NM2 in all the cell lines (**Fig. 11B**). As expected the

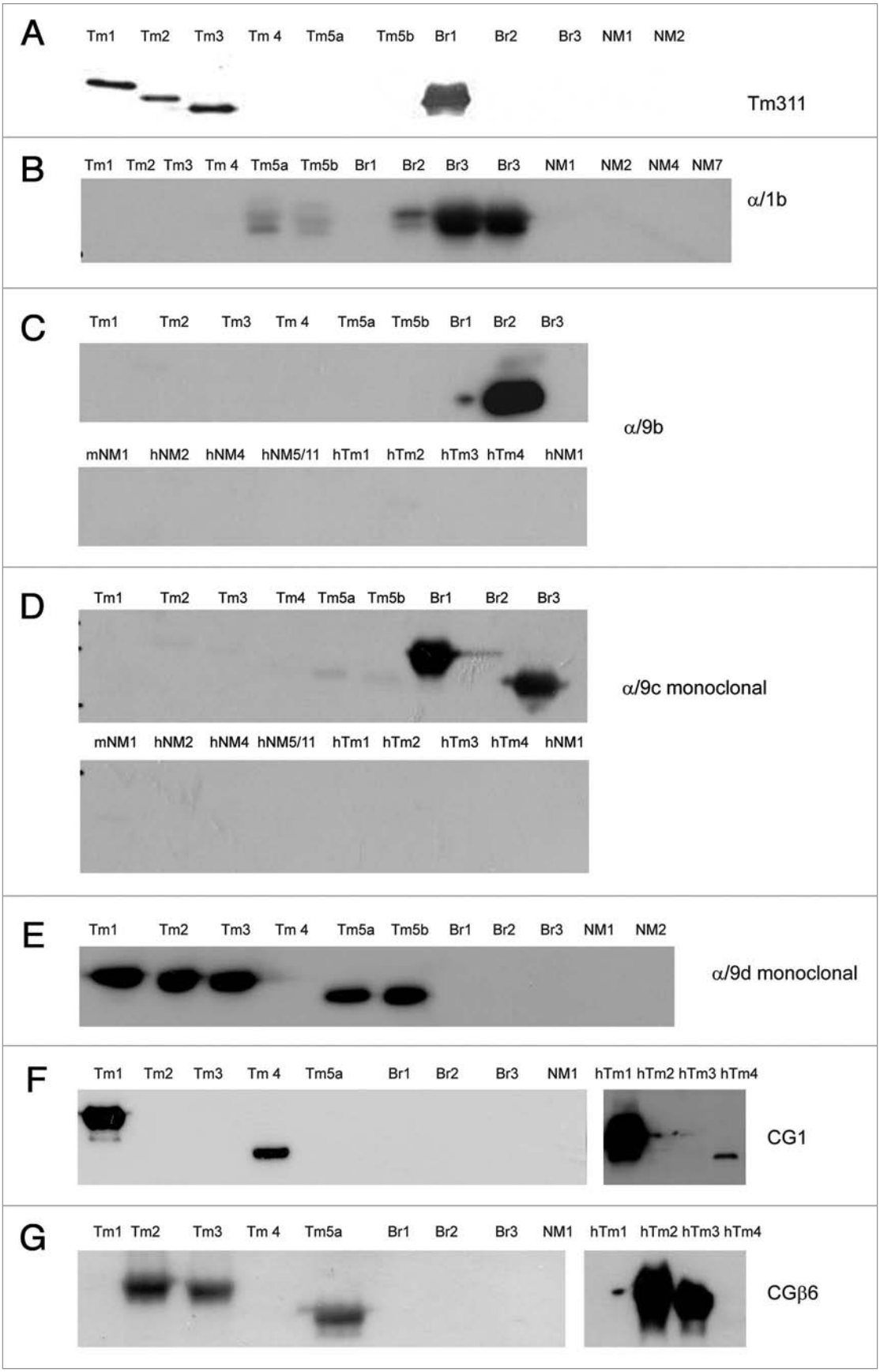

Figure 7. Evaluation of the specificity of TPM1 and TPM2 genes directed antibodies with bacterially produced Tm proteins. Protein gel blot analysis of 0.5 μg recombinant Tms probed with (A) TM311, (B) α/1b, (C) α/9b, (D) α/9c, (E) α/9d monoclonal, (F) CG1 and (G) CGβ6 antibodies.

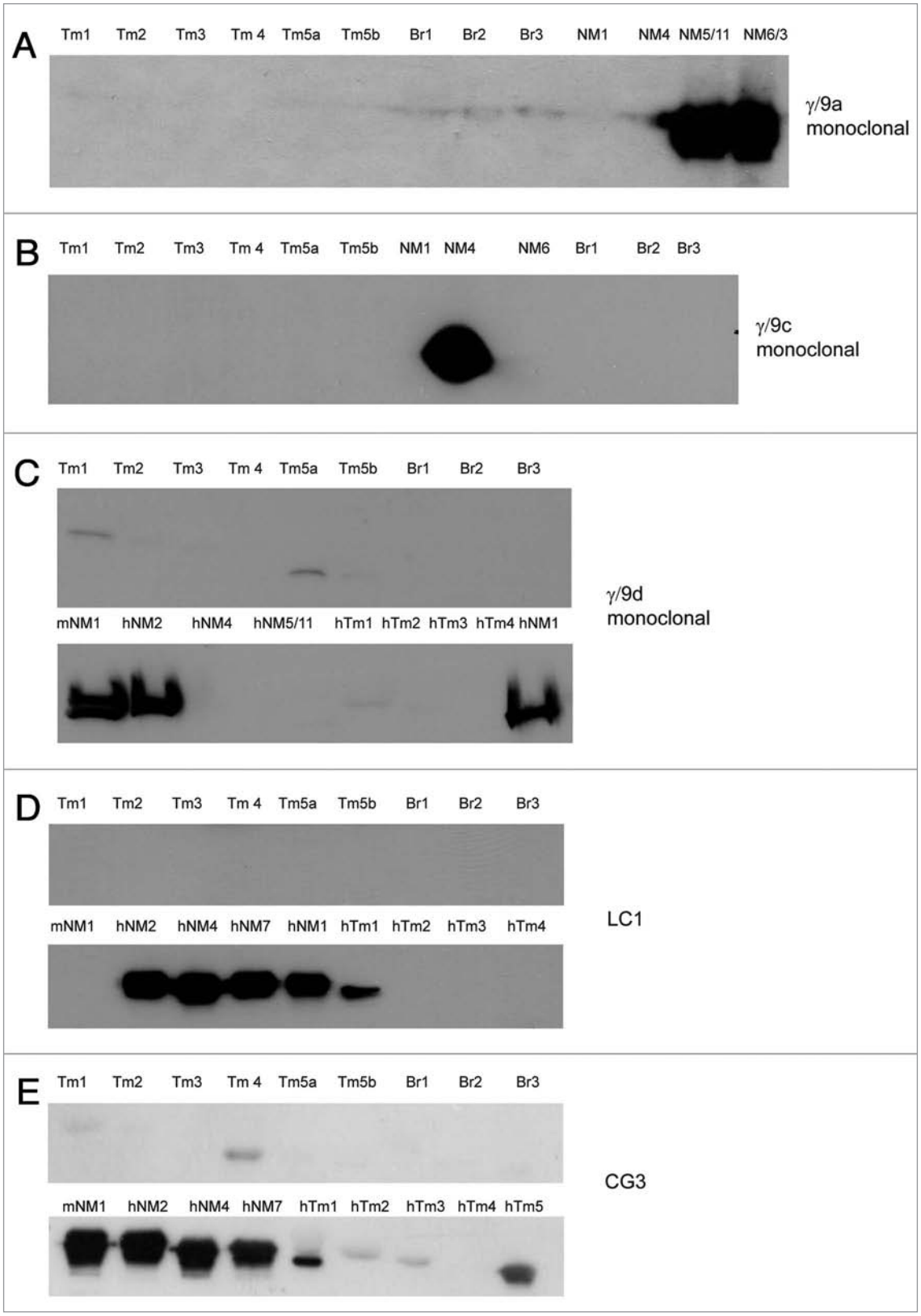

Figure 8. Evaluation of the specificity of TPM3 gene directed monoclonal antibodies with bacterially produced Tm proteins. Protein gel blot analysis of 0.5μg recombinant Tms probed with (A) γ/9a, (B) γ/9c, (C) γ/9d, (D) LC1 and (E) CG3 antibodies.

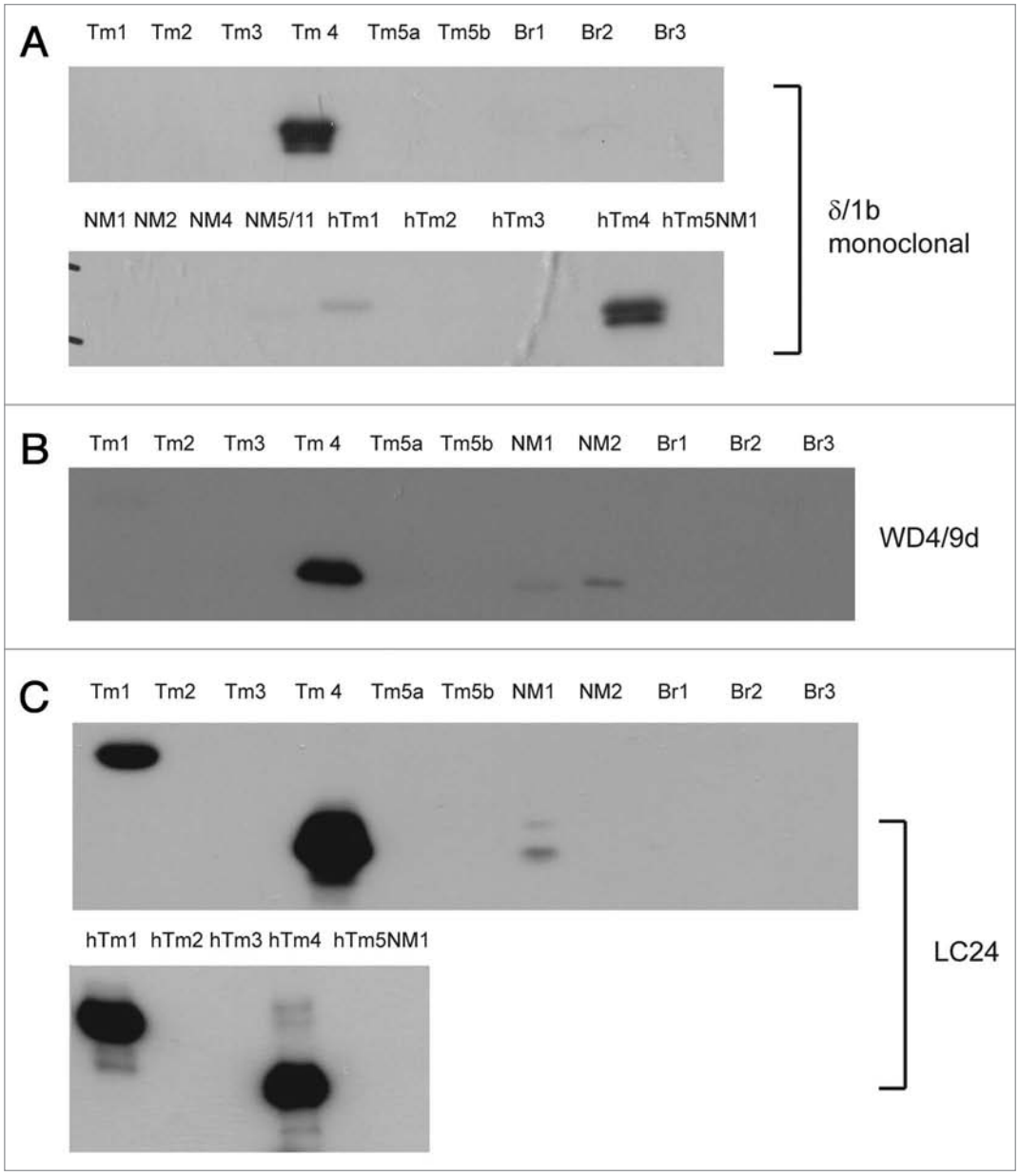

Figure 9. Evaluation of the specificity of TPM4 gene directed antibodies with bacterially produced Tm proteins. Protein gel blot analysis of 0.5 μg recombinant Tms probed with (A) δ/1b, (B) WD4/9d and (C) LC24 antibodies.

LC1 antibody, like CG3, detects a band in all the human cell lines as its epitope is in exon 1b, but CG3 also sees the same band in mouse and rat unlike LC1 which is human specific (**Fig. 11C**). The exon 9c containing isoforms identified with the γ/9c antibody were found to be undetected by protein gel blotting (data not shown). Exon 9c containing isoforms are primarily detected in central nervous system cells.[67,68]

Analysis of expression of the cytoskeletal Tm4 isoform with the δ/1b, WD4/9d and LC24 antibodies is shown in **Figure 12**. The δ/1b is very specific to Tm4 (**Fig. 9A**). In contrast, the WD4/9d detects in addition to Tm4 a HMW isoform predicted to be Tm1 (**Fig. 12B**) which is relatively abundant in the human and mouse cells, as seen with the TM311 and α/9d antibodies (**Fig. 10A and C**). Surprisingly, the LC24 antibody detects Tm4 in human and rat cells but not in the mouse fibroblasts. Further confirmation of the failure of this antibody to detect the mouse cytoskeletal Tm4 isoform is demonstrated in other mouse cells later in this report.

Total protein lysates were generated from both mouse and rat tissues and probed with the Tm antibodies described above. Even though individual tissues are comprised of many different cell types the expression of the major Tm isoforms expressed can be evaluated by protein gel blot analysis. The sheep α/1b antibody detects a prominent band of about 30 kDa in brain, liver, lung, kidney and spleen (**Fig. 13A**). Since the isoforms detected by

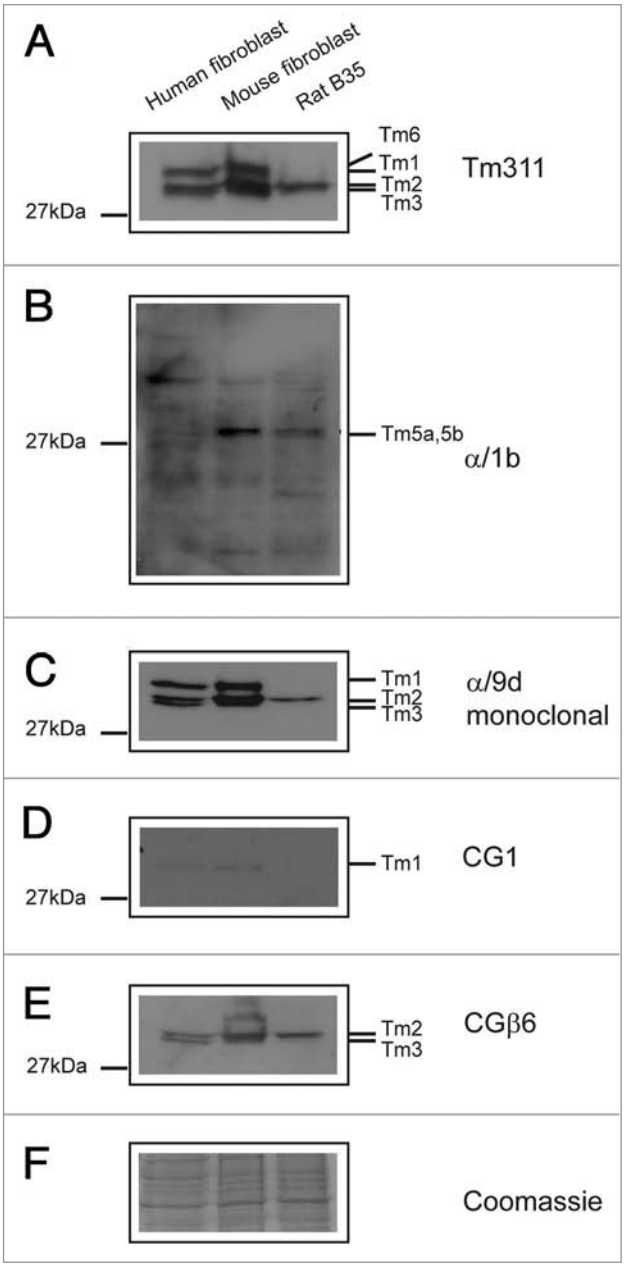

Figure 10. Expression of Tm isoforms coded by the TPM1 and TPM2 genes in three cell lines. Equal loading (20 μg) of total cellular protein isolated from human primary fibroblasts, mouse primary fibroblasts and rat neuroblastoma B35 cells, was electrophoresed on 12.5% SDS gel and individual protein gel blots probed with the (A) TM311, (B) α/1b, (C) α/9d, (D) CG1 and (E) CGβ6 antibodies. The Coomassie Blue stained gel (F) demonstrates equal protein loading.

this antibody all have similar or the same apparent molecular weights, it is difficult to distinguish whether they correspond to Tm5a, 5b or Br3. Comparison of the Tm expression profile generated with the use of the α/9d antibody shows that Tm5a/5b are most abundant in kidney, liver, lung and spleen whereas these isoforms are undetected in the adult brain at the level of sensitivity of protein gel blotting (**Fig. 13C**). Hence, we can deduce that the α/1b antibody is detecting TmBr3 in the brain. TmBr3 is one of the major isoforms expressed in mature neurons.[69-71]

The α/2a monoclonal antibody is raised against a peptide that corresponds to the unique exon 2a found only in the TPM1 gene. Exon 2a containing isoforms are the Tmsmα also known as Tm6 in addition to hTmsmα-1 and hTmskα1-1 identified in human tissues.[42] The Tmsmα (Tm6) and hTmsmα-1 are enriched in smooth muscle such as placenta, uterus[42] and stomach (**Fig. 13B**). The expression of the exon 2a containing isoform(s) is highly enriched in mouse stomach (**Fig. 13B**). Note also the slower mobility of the exon 2a containing isoform in skeletal muscle; possibly Tmskα1-1 (**Figs. 1 and 13B**).

The monoclonal antibody α/9d was raised against a peptide corresponding to the entire exon 9d of the TPM1 gene with predicted recognition of isoforms Tm1, 2, 3, 5a, 5b and 6. On a panel of mouse tissues, the expression of the HMW isoforms Tm1/6 are most highly enriched in the kidney, lung, spleen and stomach (**Fig. 13C**). Significant levels of Tm2 are detected in kidney, spleen and stomach and on a longer blot exposure (data not shown) Tm2 is also detected in lung. Expression of Tm3 was limited to the stomach sample. The LMW isoforms Tm5a/5b were enriched in the kidney, lung and spleen and to a lesser extent in the brain, liver and stomach, seen only on a longer exposure (data not shown).

The CG1 antibody, shown in **Figure 7F** to detect Tm1 but also cross-reacts with Tm4, detects a prominent HMW band in skeletal muscle most likely to correspond to βTm (**Fig. 13D**). In the kidney, lung and spleen the prominent HMW band most likely corresponds to Tm1 as Tm1 detected with the α/9d antibody is highly expressed in these tissues (**Fig. 13D** compared to **C**). The brain and heart also show a faint HMW band and both tissues also express Tm1 but at relatively lower levels (**Fig. 13D** compared to **C**). A LMW band is also detected in the kidney, lung and spleen (**Fig. 13D**). This may correspond to Tm4, as later shown, these tissues express relative high levels of Tm4 detected with the two antibodies (**Fig. 15A and C**).

There are four antibodies designed to identify Tm isoforms from the TPM3 gene. The γ/9a, γ/9c, γ/9d mouse monoclonal antibodies detect isoforms containing C-terminal exons 9a, 9c and 9d respectively. The CG3 antibody detects all the exon 1b containing isoforms. In the heart and skeletal muscle the γ/9a antibody detects a prominent band of 36

kDa corresponding to the exon 9a containing muscle isoforms from the TPM1, 2 and 3 genes with liver, lung and stomach expressing relatively lower levels of HMW isoform(s) (**Fig. 14A**). In the brain, lung, spleen and stomach the LMW, 30 kDa, cytoskeletal exon 9a containing isoforms, may correspond to either Tm5NM5/11 or NM6/3. Stomach shows a doublet at 30 kDa which may correspond to NM8 and/or 9. Tm5NM8 and 9 result from splicing of exon 9a to 9c and result in an extension of 5 additional amino acids derived from exon 9c.[33] Attempts to generate an antibody to this sequence have thus far been unsuccessful. The γ/9c antibody detects a 30 kDa band in brain, either NM4 or 7 and cross reacts with an unknown HMW band in muscle and stomach (**Fig. 14B**). The γ/9d antibody that preferentially reacts with NM1 and NM2 detects a 30 kDa band in brain, kidney, liver, lung and spleen (**Fig. 14C**) and on a longer exposure NM1/NM2 can also be detected in the heart, skeletal muscle and stomach (data not shown). The CG3 antibody detects all the LMW cytoskeletal exon 1b containing isoforms from the TPM3 gene and a 30 kDa band is seen in all the mouse tissues (**Fig. 14D**) except in the gastrocnemius muscle used in this panel, even on a longer exposure (data not shown). However, the CG3 antibody does detect a 34 kDa band in extraocular and soleus, corresponding to the α_sTm from the TPM3 gene.[65]

The TPM4 gene together with the TPM2 gene are the least alternatively spliced genes, each known to code for only two isoforms. We have generated two antibodies raised against peptides within the N-terminal end, exon 1b and the C-terminal end, exon 9d. On a panel of mouse tissues, the LMW Tm4 isoform, identified with the δ/1b antibody, is abundantly expressed in kidney, lung and spleen (**Fig. 15A**), on a longer blot exposure a band can been seen in muscle and stomach (data not shown). There is however the presence of a slightly higher MW band seen at significant levels in the liver. In order to confirm that this unknown band corresponds to Tm, we took advantage of the fact that Tm is a heat stable protein. Following the enrichment of Tm by heating the tissues to 95°C for 10 min (see Materials

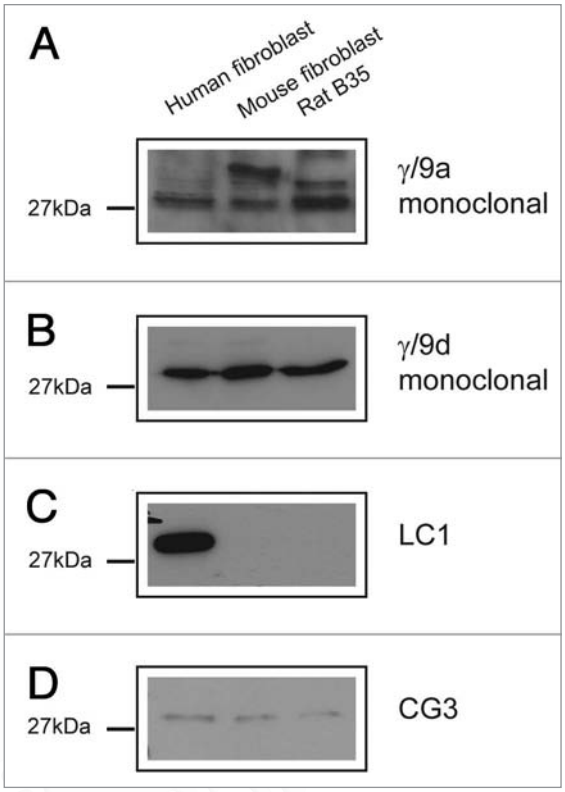

Figure 11. Expression of Tm isoforms coded by the TPM3 gene in three cell lines. Equal loading (20 μg) of total cellular protein isolated from human primary fibroblasts, mouse primary fibroblasts and rat neuroblastoma B35 cells, was electrophoresed on 12.5% SDS gels and individual protein gel blots probed with the (A) γ/9a, (B) γ/9d, (C) LC1 and (D) CG3 antibodies.

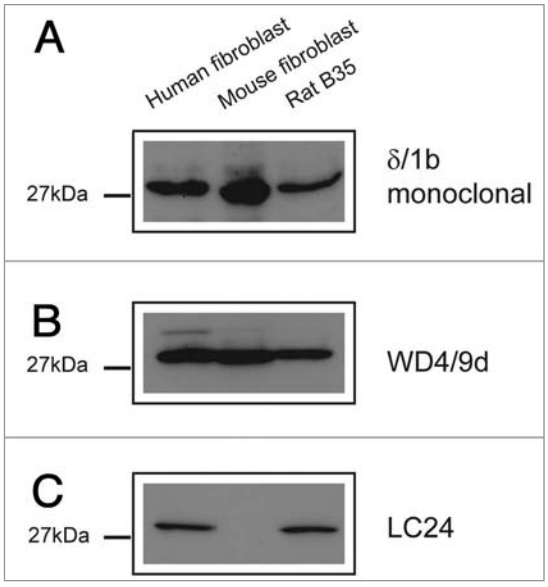

Figure 12. Expression of Tm isoforms coded by the TPM4 gene in three cell lines. Equal loading (20 μg) of total cellular protein isolated from human primary fibroblasts, mouse primary fibroblasts and rat neuroblastoma B35 cells, was electrophoresed on 12.5% SDS gels and individual protein gel blots probed with the (A) δ/1b, (B) WD4/9d and (C) LC24 antibodies.

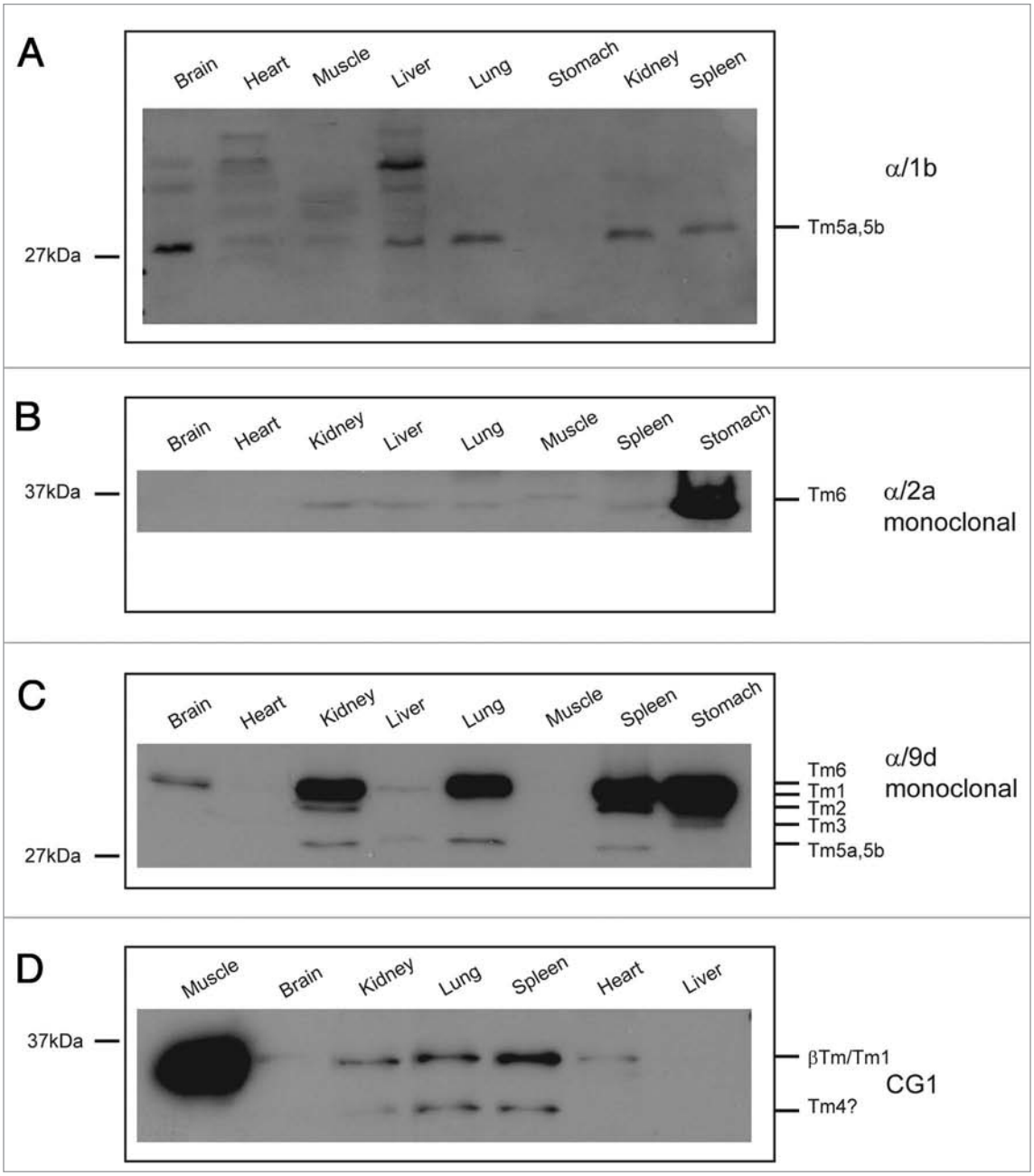

Figure 13. Expression profile of Tm isoforms coded by the TPM1 and 2 genes in a panel of mouse tissues. Equal loading (20 μg) of total cellular protein isolated from different mouse and rat tissues was electrophoresed on 12.5% SDS gels and individual protein gel blots probed with the (A) α/1b, (B) α/2a, (C) α/9d and (D) CG1 antibodies.

and Methods) we found that the HMW bands seen in kidney and liver were now absent (**Fig. 15B**). We conclude that δ/1b is detecting non-Tm proteins in mouse tissues. WD4/9d predominantly detects the LMW Tm4 isoform in all tissues examined even in brain and muscle on a longer exposure (not shown) (**Fig. 15C**). The LC24 was shown to detect both the human and rat Tm1 and Tm4 recombinant proteins (**Fig. 9B**) however it failed to detect the Tm4 in mouse protein lysates (**Fig. 12C**). Similarly, in the case of the mouse tissues the LC24 does not detect the LMW cytoskeletal Tm4 isoform (**Fig. 15D**). However, a HMW band is seen which in mouse we predict to be βTm whereas in liver, lung and spleen it may correspond to Tm1 (**Fig. 15D**). Surprisingly, in the rat tissues the LC24 antibody detects primarily Tm4 and a HMW isoform in muscle (**Fig. 15E**).

Some of the Tm antibodies described above have been successfully used to perform immunoprecipitations. These include LC1 and WSα/9c,[16] anti-sheep α/2a and CG1 [72] and TM311.[73]

In summary, one of the major limitations in evaluating the Tm isoform

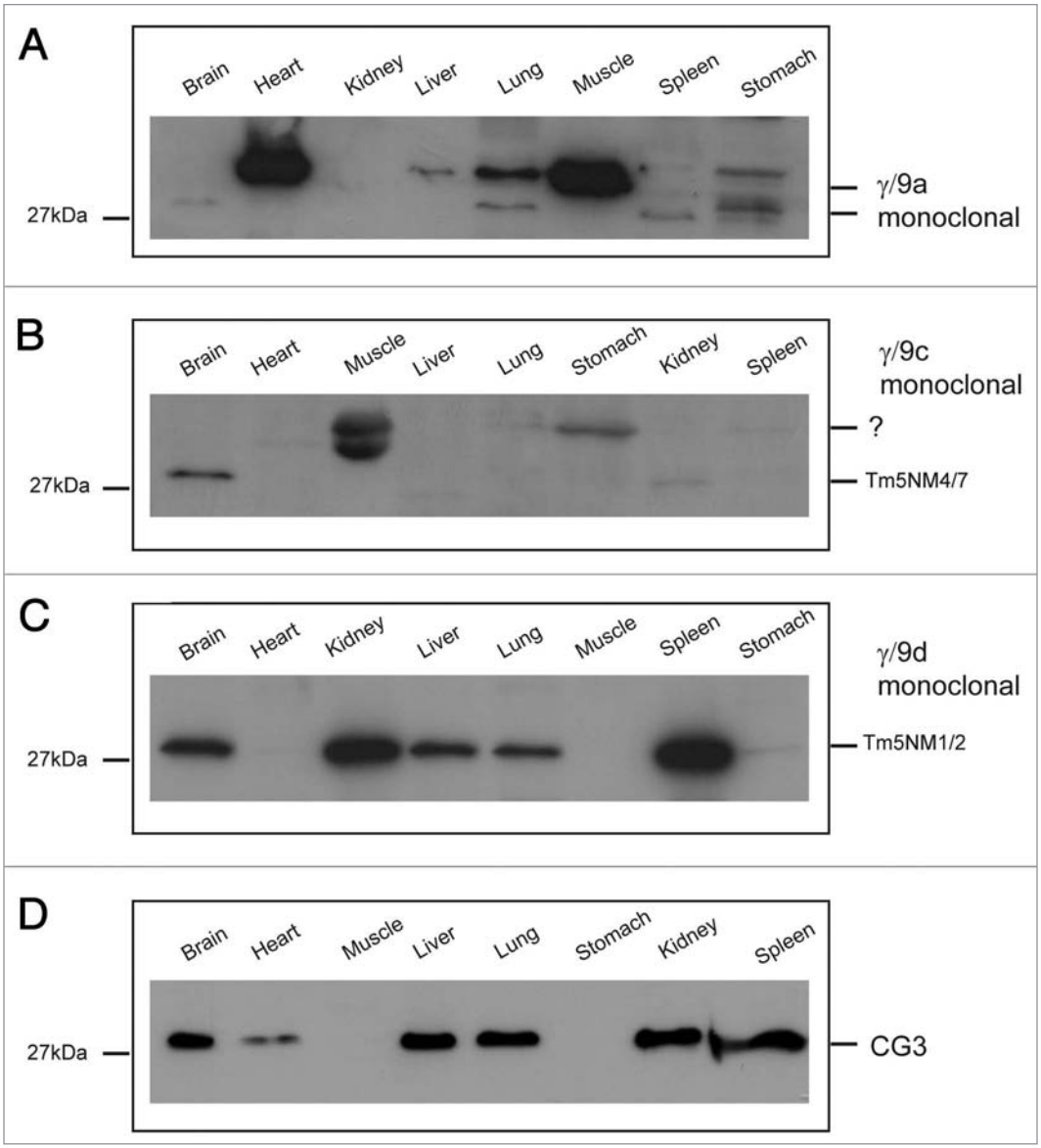

Figure 14. Expression profile of Tm isoforms coded by the TPM3 gene in a panel of mouse tissues. Equal loading (20 μg) of total cellular protein isolated from different mouse tissues was electrophoresed on 12.5% SDS gels and individual protein gel blots probed with the (A) γ/9a, (B) γ/9c, (C) γ/9d and (D) CG3 antibodies.

composition in either cell lines or tissues is the similarity in the apparent molecular weights when 1-D gel electrophoresis is performed. 2-D electrophoresis may allow for the identification of additional LMW isoforms as some display distinct pI values (**Table 4**). In some cases quantitative PCR will be the best methodology to assess the presence and quantity of different Tm isoforms, albeit at the mRNA level.

Tm isoforms mark distinct actin filament populations. Distinct intracellular subcellular sorting of different Tm isoforms has to date been documented in various cell model systems both in vitro and in vivo (reviewed in refs. 1 and 7). This observed sorting of Tm isoforms implies the existence of distinct populations of actin filaments, marked by Tm isoforms, influencing the dynamics and organization properties of the filaments.[1] In order to further evaluate the performance of the Tm antibodies described above, immunofluorescence staining was performed on two cell lines, normal and tumor cells, mouse primary embryonic fibroblasts and the neuroblastoma B35 cells, respectively. The choice of cells is based on the fact that these cells (1) display in the case of the mouse primary fibroblasts, a well organized actin cytoskeleton,[60] (2) exhibit specific structures such as ruffling membrane and filopodial, (3) according to **Figures 10–12** express various Tm isoforms and (4) represent two commonly used cell types. At the level of resolution of an epifluorescence microscope, a clear localization of Tm to actin stress fibers was far more evident in the normal mouse primary embryonic fibroblasts than in the neuroblastoma B35 cells (compare **Figs. 16–18 to 19–21**). Subtle

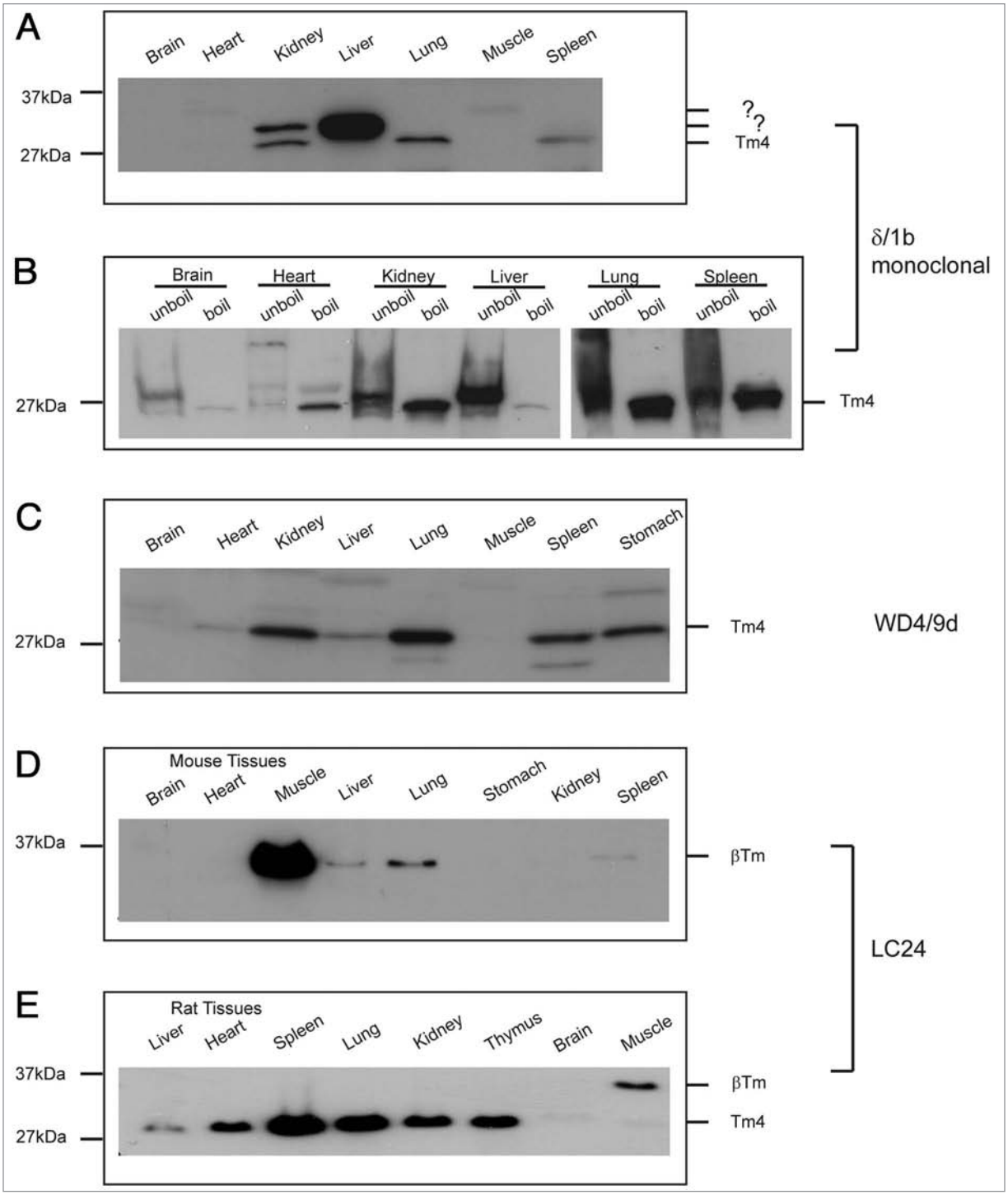

Figure 15. Expression profile of Tm isoforms coded by the TPM4 gene in a part of mouse and rat tissues. Equal loading (20 μg) of total cellular protein isolated from different mouse (A–D) and rat (E) tissues was electrophoresed on 12.5% SDS gels and individual protein gel blots probed with the (A and B) δ/1b, (C) WD4/9d and (D and E) LC24 antibodies. For part (B) the tissues were either unboiled or boiled in order to enrich for Tm.

differences among the Tm antibodies consist primarily on whether the staining for the Tms extends to the periphery of the cell and the intensity of staining, reflecting the levels of expression. Comparison of the staining patterns of the TM311 (Tm1, 2, 3, 6, Br1) with the α/1b (Tm5a, 5b, Br2, Br3) antibodies demonstrates the diminished staining of TM311 at the periphery of the cell whereas the α/1b extends and is enriched at the cell periphery, seen in both the mouse fibroblasts (**Fig. 16K** compared to **L**, arrows) and rat B35 cells (**Fig. 19K** compared to **L**, arrows). This observed staining strongly suggests that Tm5a/5b are peripherally enriched isoforms and this correlates with the apical enrichment of these isoforms in

epithelial cells.[74] Similarly, the α/9d antibody which detects the HMW Tms, 1, 2, 3, 6 and the LMW Tm5a, 5b is also seen to extend to the cell periphery in mouse fibroblasts (**Fig. 16M**, arrow) and B35 cells (**Fig. 19M** and arrow) although not as dramatically as for the α/1b antibody. The CG1 (Tm1) antibody detects prominent stress fibers in the mouse fibroblasts (**Fig. 16D and N**, arrow) and staining is absent in the B35 cells (**Fig. 19N**). The CGβ6 (Tm2, 3) antibody is seen to detect stress fibers with a striated appearance most prominent in the B35 cells (**Figs. 16E and 19E**, see inset). Both the γ/9d (Tm5NM1, NM2) and CG3 (all TPM3 cytoskeletal products) antibodies detect stress fibers and no enrichment of staining is seen at the cell periphery (**Fig. 17E, F and 20E and F**, arrows). The WD4/9d antibody identifies stress fibers best seen in the mouse fibroblasts as compared to the B35 cells (**Fig. 18E** compared to **21E**) and staining is reduced at the cell periphery, most apparent in the mouse fibroblasts (**Fig. 18E**, arrow). Finally, the LC24 antibody exhibits stress fiber staining in the human immortal fibroblasts and B35 cells (**Figs. 18F and 21F**) while no staining was detected in the mouse fibroblast cells (data not shown). There is a significant difference in the pattern of LC24 staining seen between the human immortal cells and the B35 cells, with a more even intensity of staining seen in the immortal cells while in the B35 cells the staining is significantly diminished at the cell periphery (**Fig. 18F** compare to **21F**, arrows).

In conclusion, at the level of resolution of an epifluorescence microscope the major differences among the various Tm antibody staining patterns was their enrichment at the cell periphery. In addition, subtle differences among the different cell types, normal and immortal fibroblasts, were observed.

Subcellular localization of individually tagged Tm isoforms. One of the major drawbacks of using the Tm antibodies to evaluate the subcellular localization of Tms is that the majority of antibodies do detect several isoforms. In order to investigate the intracellular localization of individual Tm isoforms, specific isoforms have been cloned into plasmids containing YFP or GFP (yellow or green fluorescence protein) or hemagglutinin (HA) tags (**Table 5**). Surprisingly, the addition of a 27 kDa YFP or GFP protein to the N-terminal end appears not to interfere with the head-to-tail association of the Tm dimers, as these tagged proteins are quite capable of forming polymer like structures that colocalize with actin filaments.[75] In addition, YFP.Tm5NM1 has been shown to have a direct role in cell migration as its expression in Tm5NM1 null cells rescued the phenotype further demonstrating the functionality of tagged Tms.[76] The concern encountered with transfection studies is the fidelity of sorting, as overexpression of these tagged isoforms may lead to the saturation of pools and hence inappropriate subcellular localization. However, recent studies in U2OS cells have shown remarkable fidelity of sorting of these tagged Tms.[21]

Overexpression and targeted gene siRNA knockdown in vitro based methods to study Tm isoform specific function. In order to evaluate the biological function of the different Tm isoforms, overexpression (**Table 6**) and siRNA knockdown (**Table 7**) methodology has been employed. The overexpression studies have shed light on the importance of Tm isoforms in defining the organizational properties and molecular composition of actin filaments ultimately influencing the overall morphology of cells.[16,21,77-80] In addition, overexpression studies complemented with targeted gene siRNA knockdown experiments have demonstrated the importance of specific Tm isoforms in regulating the structure and dynamic properties of focal adhesions.[76,81] Finally, recent siRNA knockdown experiments have played an important role in establishing the nonredundant roles of Tm isoforms in stress fiber assembly.[21]

The development of mouse models to study tropomyosin. The primary objective for the generation of Tm gene modified mice has been to study the disease process of various human myopathies. Mutations in both the TPM2 and TPM3 genes are associated with diseases of skeletal muscle such as nemaline myopathy, distal arthrogryposis and most recently "cap" disease. TPM1 gene mutations have been linked to familial hypertrophic cardiomyopathy and dilated cardiomyopathy (reviewed in refs. 82–84). The current mouse models that recapitulated these human myopathies are listed in **Table 8**. It is noteworthy to mention that

Table 4. Theoretical isoelectric focusing points

Tm isoform	Apparent molecular weights on SDS PAGE gels (kDa)	Theoretical pI
Tm6	40	4.70
Tm1	38	4.63
Tm2	36	4.69
Tm3	34	4.69
Tm5a	30	4.74
Tm5b	30	4.75
TmBr1	34	4.69
TmBr2	30	4.84
TmBr3	30	4.74
Tm5NM1	30	4.72
Tm5NM2	30	4.73
Tm5NM5/1	30	4.71
Tm5NM6/3	30	4.69
Tm5NM4	30	4.79
Tm5NM7	30	4.81
Tm5NM8	30	4.71
Tm5NM9	30	4.69
Tm4	30	4.65

ExPASy Proteomics server (http://expasy.org/tools/pi_tool.html) was utilized to determine the theoretical pI value.

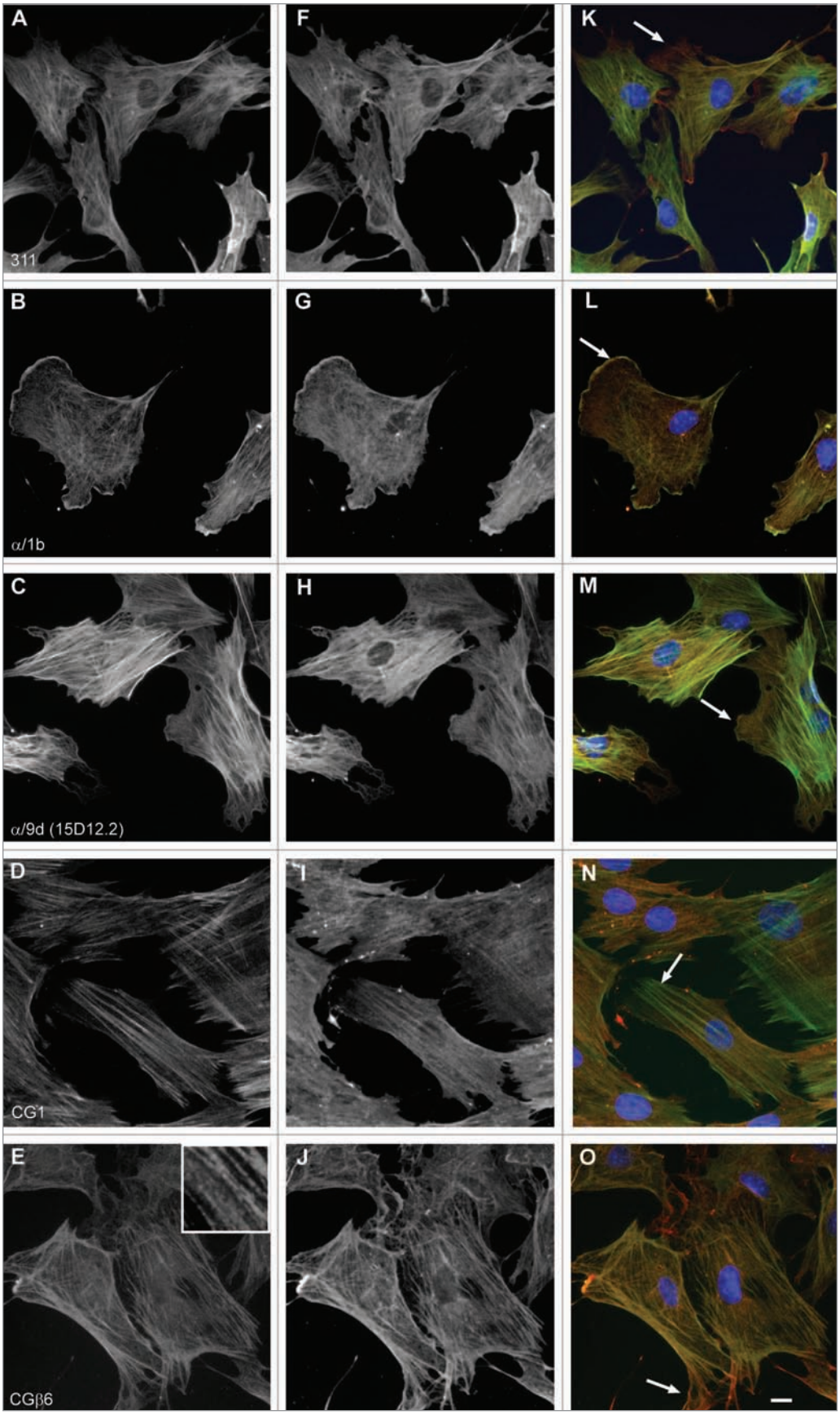

Figure 16. For figure legend, see page 155.

Figure 16 (See opposite page). Immunofluorescence staining of mouse primary embryonic fibroblasts with antibodies directed to Tm isoforms coded by the TPM1 and TPM2 genes. (A–E) Tms in green, (F–J) γactin in red and (K–O) merge images. Nuclei were visualized with DAPI. Arrows in (K–O) denote the cell periphery and in (N) stress fibers. Bar = 10 μm.

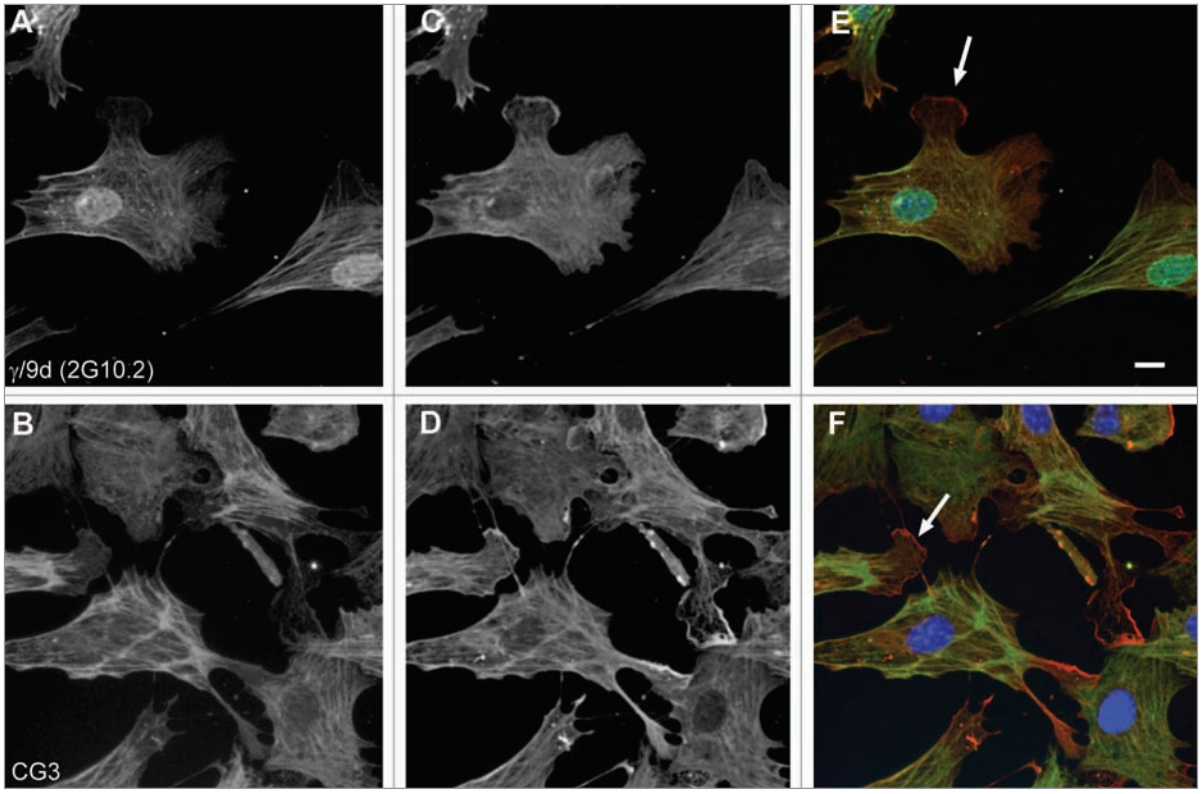

Figure 17. Immunofluorescence staining of mouse primary embryonic fibroblasts with antibodies directed to Tm isoforms coded by the TPM3 gene. (A and B) Tms in green, (C and D) γactin in red and (E and F) merge images. Nuclei were visualized with DAPI. Arrows in (E and F) denote the cell periphery. Bar = 10 μm.

although the Tm mutations linked to human diseases are known to exhibit clinical abnormalities mostly restricted to the heart and skeletal muscle, these mutations are present in exons shared by numerous other Tm isoforms (see **Tables 2 and 3**).[85] All known cardiomyopathies carry mutations in Tm isoforms specific to the central nervous system including TmBr1, TmBr2 and TmBr3. In the skeletal muscle myopathies, mutations in the TPM3 gene occur in exons 3, 5 and 8 which are common to all the Tm isoforms (Tm5NM1-11) encoded by this gene. Hence, it is possible that these patients may also display uncharacterized pathologies in organs other than skeletal muscle or the heart. Unfortunately, in the case of the mouse models, it is not possible to address the impact that these Tm mutations may have in other organs since the altered Tms are expressed in a tissue-specific manner. The human skeletal muscle actin or the cardiac specific α-myosin heavy chain promoters were used to generate these transgenic mice.

Various other genetically modified mouse models have been generated primarily to dissect the functional differences among the isoforms and are listed in **Table 9**. The knockout mouse models reveal that the Tm genes are essential for life as deletion of the TPM1, 2 and 3 genes are embryonic lethal and offer further evidence for the non-redundant nature of the mammalian Tm genes.[84,86-89] To date, two transgenic mouse models exist that have overexpression of the cytoskeletal Tm isoforms, Tm3 and Tm5NM1, in major organs as the transgene is driven by the human β-actin promoter.[90] The Tm3 transgenic mice demonstrate dystrophic features perhaps due to increase sensitivity to contractile-induced stress.[65,91] In the case of the Tm5NM1/NM2 knockout mice, lack of these isoforms leads to T-tubule dysmorphology and altered skeletal muscle function.[92] Both the Tm3 and Tm5NM1 transgenics together with the complementary Tm5NM1 knockout mice have become valuable resources for the isolation of primary embryonic cells such as neuronal and fibroblast cells. Primary cortical neurons isolated from these mice have revealed the importance of Tm isoforms in the development and maintenance of neuronal morphogenesis, previously only inferred from subcellular localization studies.[16,80,93]

Materials and Methods

Antibodies. Table 3 lists the Tm antibodies used in this report. The most suitable dilutions for protein gel blot analysis for the affinity purified mouse monoclonal

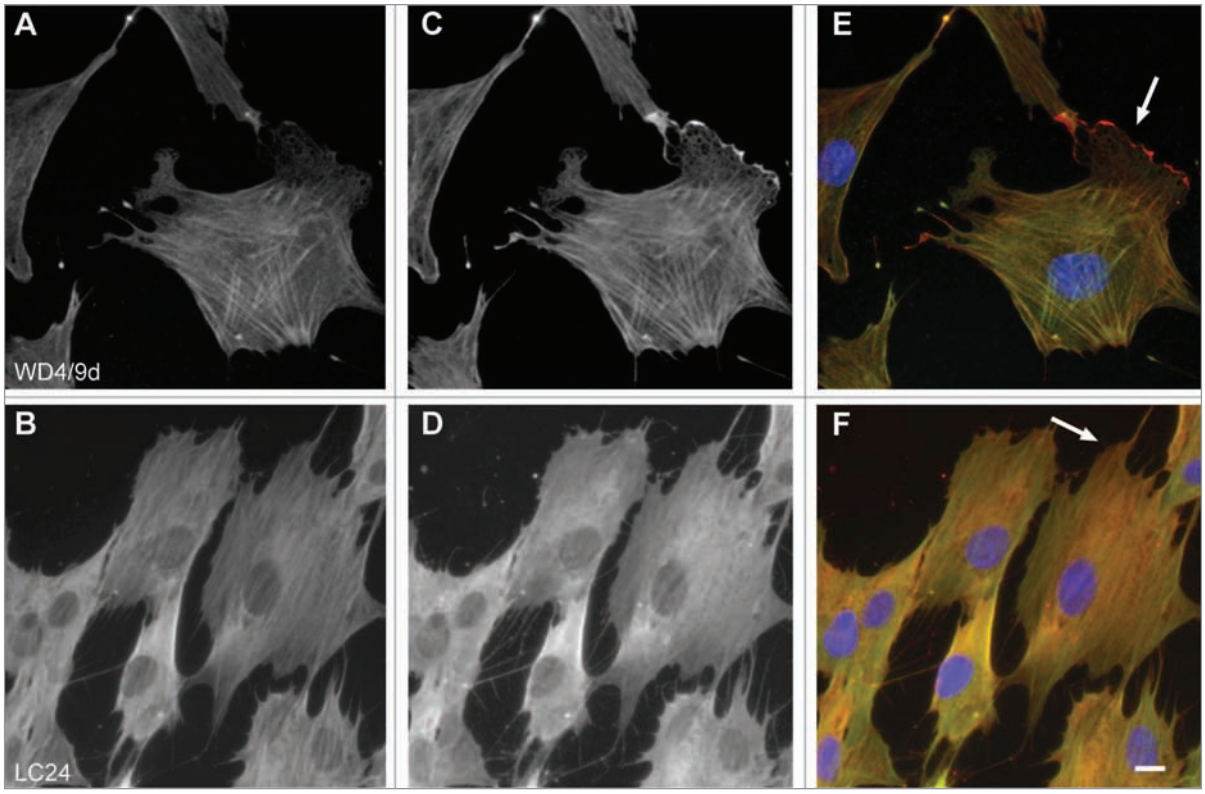

Figure 18. Immunofluorescence staining of fibroblasts with antibodies directed to Tm isoforms coded by the TPM4 gene. (A and B) Tms in green, (C and D) γactin in red and (E and F) merge images. Nuclei were visualized with DAPI. (A, C and E) correspond to mouse fibroblasts and (B, D and F) human immortalized fibroblast cells. Arrows in (E and F) denote the cell periphery. Bar = 10 μm.

antibodies were as follows: TM311 (Cat# T2780, Sigma) at 1:500; α/2a at 1:1,000; α/9b; CH1 (Cat# T9283, Sigma) at 1:50; γ/9a at 1:500; γ/9c at 1:500; γ/9d at 1:500 and δ/1b at 1:1,000. We used the hybridoma supernatant for the following mouse monoclonal antibodies, CG1 at 1:100, CGβ6 at 1:100; LC1 at 1:250; CG3 at 1:250, LC24 at 1:100 and α/9c (clone#554, kind gift from Jim Lessard, Cincinatti Children's Hospital Medical Center, Ohio, USA) at 1/500 dilutions. The primary rabbit polyclonal WD4/9d was used at 1:500 dilution. The α/9d, γ/9a, γ/9c and γ/9d mouse monoclonal antibodies were generated by initial synthesis of a peptide conjugated to diphtheria toxin (Millipore). The α/2a, α/9b, γ/9a and δ/1b mouse monoclonal antibodies were generated by initial synthesis of a peptide conjugated to keyhole limpet hemocyanin (KLH) (ProMab Biotechnologies, Inc., CA USA). The secondary antibodies for protein gel blotting were anti-rabbit IgG horseradish peroxidise-linked whole antibody (from donkey) (Cat# NA9341ML) and anti-mouse IgG horseradish peroxidise-linked whole antibody (from sheep) (Cat# NA9311ML) (GE Healthcare). For immunofluorescence staining essentially the same dilution as that used for protein gel blotting was employed. The actin cytoskeleton was visualized by double immunostaining with the anti-sheep γactin antibody at 1:1,000 dilution.[60] The secondary antibodies were alexa Fluor® 555 goat anti-rabbit IgG (H + L) (Cat# A-21428), alexa Fluor® 488 goat anti-rabbit (Cat# A-11008), alexa Fluor® 555 goat anti-mouse IgG (H + L) (Cat# A-21422) and alexa Fluor® 488 goat anti-mouse IgG (H + L) (Cat# A11001) and alexa Fluor® 555 donkey anti-sheep IgG (H + L) (Cat# A21436) (Invitrogen).

Recombinant Tms expression constructs. The pPROEX HT prokaryotic expression system (Gibco BRL, Invitrogen; Melbourne, Australia) or the pET bacterial plasmid systems were used for the production of recombinant Tm isoform proteins. The Tm cDNAs (Tm2, 3, 5a, 5b and NM1 in pGEX plasmids) were originally supplied by D. Helfman and hTm1, hTm2, hTm3 and hTm4 in the pET bacterial plasmid system were supplied by J.J.C Lin. The remaining Tm isoforms made in-house were PCR amplified and cloned into the pPROEX HT vector using restriction enzymes engineered into the PCR primers. All Tm constructs were verified by sequencing. A protocol for induction of recombinant protein was as previously described in reference 60.

Cell culture. Human immortalized embryonic fibroblasts were a gift from the Weinberg laboratory and cultured as previously described in reference 96. The primary mouse embryonic fibroblasts were isolated and cultured as previously described in reference 60. The rat neuroblastoma B35 cells were cultured as previously described in reference 16. The cells were maintained in DMEM, 10% fetal calf serum at 37°C humidified atmosphere with 5% CO_2.

Enrichment of Tm protein by heat. Mouse tissues were homogenized in

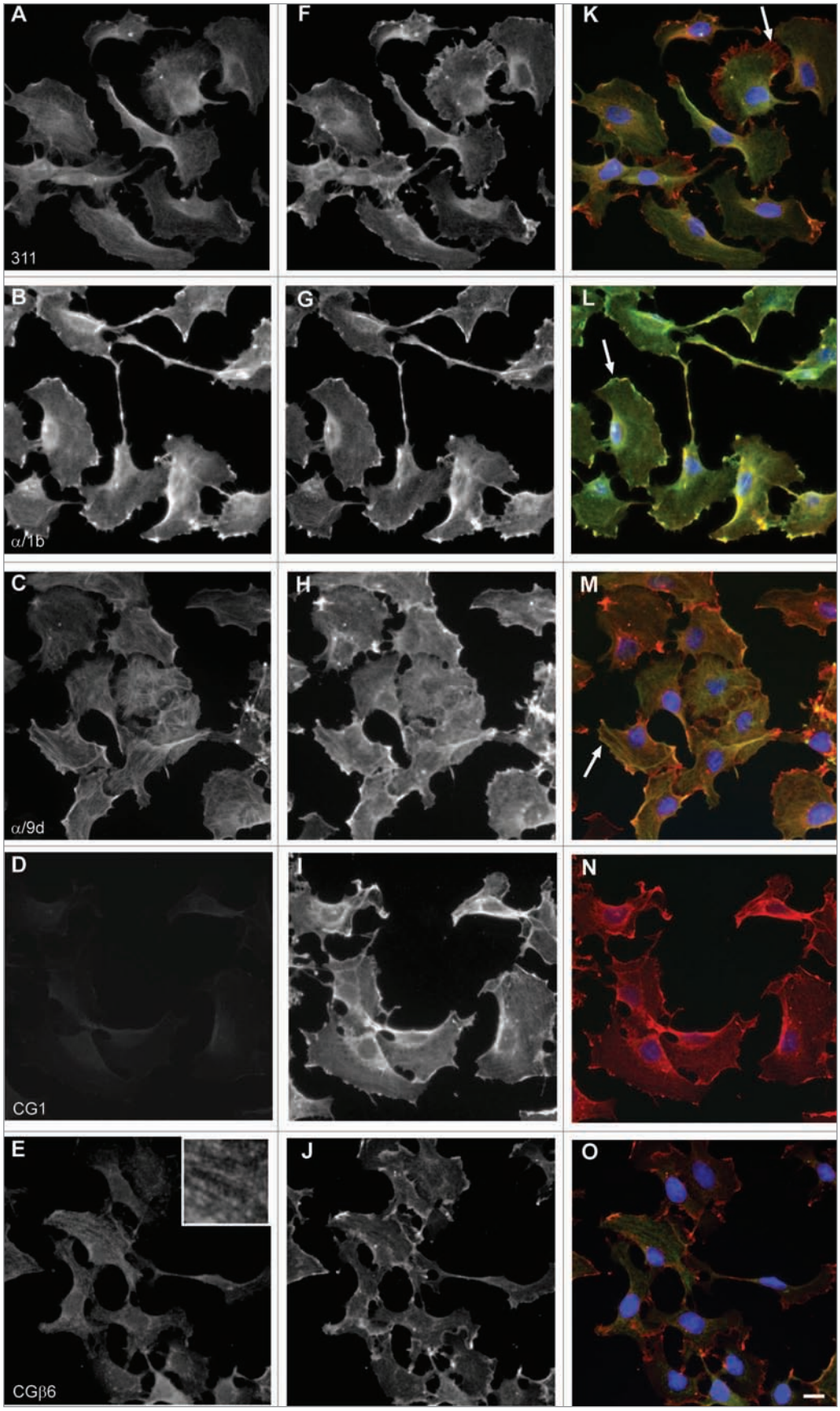

Figure 19. Immunofluorescence staining of B35 cells with antibodies directed to Tm isoforms coded by the TPM1 and TPM2 genes. (A–E) Tms in green, (F–J) γactin in red and (K–O) merge images. Nuclei were visualized with DAPI. Arrows in (K–M) denote the cell periphery. Bar = 10 μm.

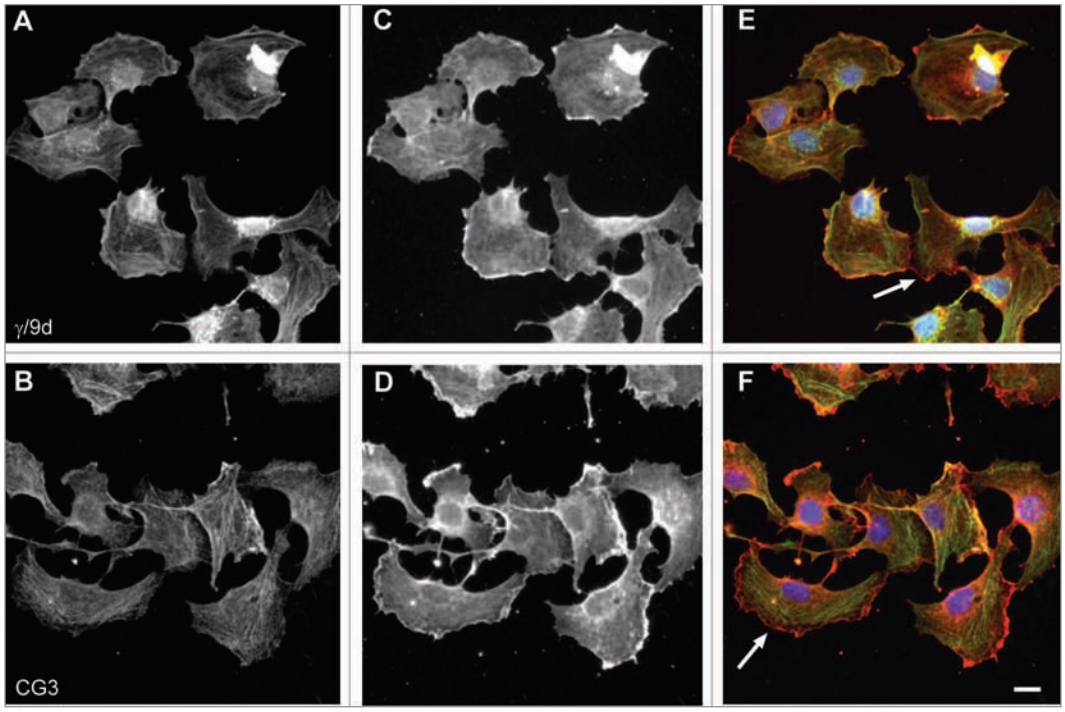

Figure 20. Immunofluorescence staining of B35 cells with antibodies directed to Tm isoforms coded by the TPM3 gene. (A and B) Tms in green, (C and D) γactin in red and (E and F) merge images. Nuclei were visualized with DAPI. Arrows in (E and F) denote the cell periphery. Bar = 10 μm.

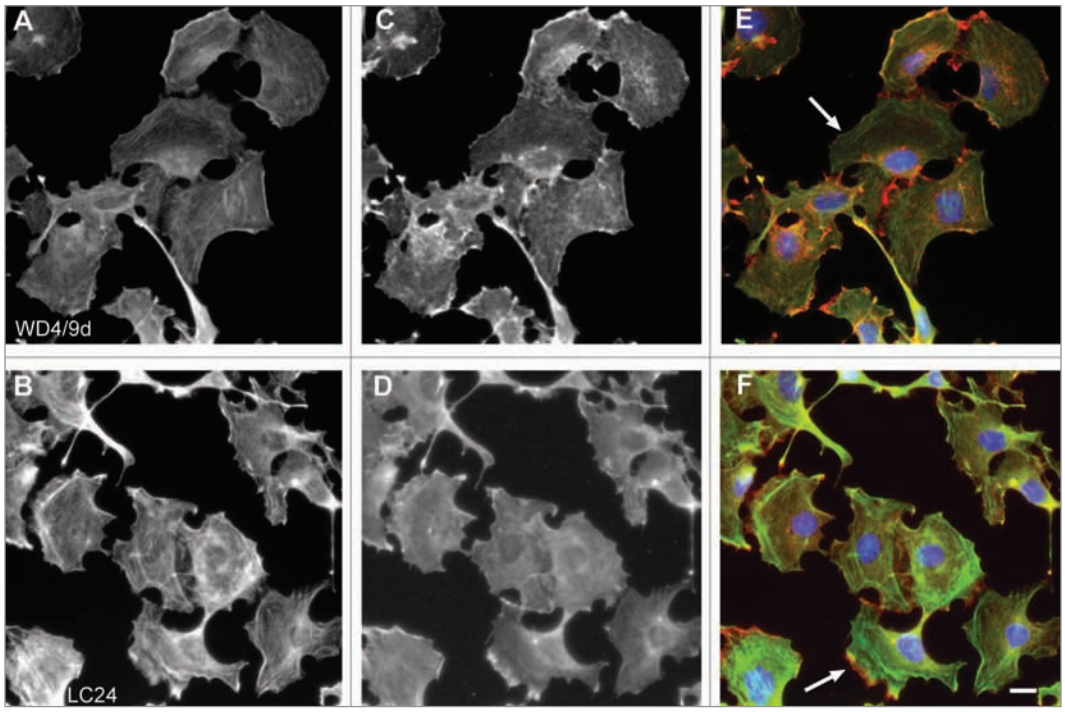

Figure 21. Immunofluorescence staining of B35 cells with antibodies directed to Tm isoforms coded by the TPM4 gene. (A and B) Tm in green, (C and D) γactin in red and (E and F) merge images. Nuclei were visualized with DAPI. Arrows in (E and F) denote the cell periphery. Bar = 10 μm.

50 mM Tris-Cl, pH 8.0, a sample representing the unheated one was taken and the remainder heated to 95°C for 10 min. After cooling on ice the samples were centrifuged for 10 min at 13,000 g and the supernatant transferred to a clean eppendorf. The chloroform/methanol extraction protocol previously described in reference 97, was used to concentrate the samples. The protein pellets were solubilized in RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA,

Table 5. Expression of fluorescently tagged Tm isoforms to study intracellular localization

Tm isoforms	Cell type and intracellular localization	Reference
HA-Tm1, 2, 3, 4, 5NM1 GFP-Tm1, 2, 3, 4, 5NM1	Both HA and GFP tagged Tms were found in stress fibers and extended into the lamellipodia and filopodia of spreading cells. HA-Tm4 and Tm5NM1 are more enriched in the lamellipodia region than the other Tms. Rat mammary adenocarcinoma MTLn3 and REF cells.	104
GFP-muscle αTm, Tm1, Tm2, Tm3, Tm4, Tm5a, Tm5NM1	All except for Tm4 incorporated into sarcomeres. Incorporation of Tm4 into sarcomeres was dependent on the presence of the other Tms. Neonatal rat cardiomyocytes.	105
YFP-Tm3, Tm5NM1 GFP-Tm5b	Tm3 was found to be less abundant at the cell periphery compared to Tm5b and Tm5NM1. C2C12 mouse myoblasts.	75
YFP-Tm1, 3, 4, 5NM1, 5NM2 GFP-Tm2	Each isoform associated with transverse arcs and ventral stress fibers. Within dorsal fibers, Tm2 was found along the entire fiber, whereas Tm1, Tm5NM1 and Tm5NM2 concentrated at the distal ends. Tm3 and Tm4 localized proximally to focal adhesions. Live cell imaging of newly forming focal adhesion shows the presence of Tm5NM2 followed by Tm2. Human osteosarcoma U2OS cells.	21

Table 6. Summary of genetically modified cell lines

Parental Line	Tm isoform	Reference
B35 rat neuroepithelial	Rat TmBr1, stable overexpression	81
B35 rat neuroepithelial	Rat TmBr3, stable overexpression	16, 81
B35 rat neuroepithelial	Rat Tm3, stable overexpression	79, 106
B35 rat neuroepithelial	Human Tm5NM1, stable overexpression	16, 76, 106, 107
RAW264.7 mouse macrophage	Mouse Tm2, stable overexpression	78
RAW264.7 mouse macrophage	Mouse Tm3, stable overexpression	78
RAW264.7 mouse macrophage	Mouse Tm4, stable overexpression	77
Primary Mouse Embryonic Fibroblasts	Mouse Cells isolated from the 129-*Tpm3*$^{tm1(neo;\Delta 9d)Pgun}$ Lack Tm5NM1 and NM2	76, 90

1% Nonidet P-40, 0.1% SDS, 1% Na Deoxycholate) followed by estimation of the protein concentration.

Immunofluorescence staining. For all the antibodies reported here, cells were washed gently three times with warm PBS, to avoid disrupting delicate structures such as ruffling membranes and/or filapodia, fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS and permeabilized with chilled methanol for 15 min. Non-specific binding was reduced by incubation with 2% fetal bovine serum for 1 h. Primary antibodies were diluted in PBS and incubated at room temperature for 1 h followed by three washes with 2% fetal bovine serum. The secondary antibodies, diluted at 1:1,000, were incubated in the dark for 1 h, washed three times with PBS and coverslip mounted using FluorSave™ Reagent (Calbiochem). If using this type of mounting media the coverslip must be completely sealed with nail polish. Nuclei were visualized with DAPI staining. Coverslips were examined using the AxioSkop 40 microsope (Carl Zeiss).

Gel electrophoresis and immunoblotting. The preparation of total protein lysates from the cell lines and different mouse tissues and analysis by SDS PAGE gel electrophoresis is essentially as described in reference 60. Protein concentration was estimated using a BCA protein detection kit (Pierce; Rockford, IL) and equal protein loading was examined by staining the protein gel blots with 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol and 10% (v/v) acetic acid. A total of 0.5 μg of recombinant proteins, 10 μg of cell line and 20 μg of animal tissues lysates were loaded. Following the transfer of proteins to Immobilon-P PVDF membrane (Millipore Corp., Bedford, MA) for 2 h at 80 V, non-specific binding on the blot was blocked with 5% low-fat skim milk in TBS (100

Table 7. Summary of published knock-down siRNA sequences

Tm isoform	Gene/exon	Species	Sequence	Reference
Tm5NM1, Tm5NM2	TPM3 gene: exon 9d	Human	siRNA: 5'-AAA GAG GAG CAC CTC TGT ACA tt-3' (sense)	76, 106
Tm2/3	*Tpm1*: exons 1a and 2b	Mouse	siRNA1: 5'-AAC UCA AGG GCA CUG AAG Att-3' (sense) siRNA2: 5'-GAU GCU GAA GCU CGA CAA Att-3' (sense)	78
Tm4	*Tpm4*: 3' UTR	Mouse	siRNA1: 5'-CCC AGA GCA AAA AUU AAC Att-3' (sense) siRNA 2: 5'-CCU ACU CUG UUC UUU ACG Utt-3' (sense)	77
Tm1	TPM2	Human	5'-AAG CAC ATC GCT GAG GAT TCA-3'	21
Tm2/3	TPM1	Human	5'-AAG CTG GAG CTG GCA GAG AAA-3'	21
Tm5NM1/2	TPM3	Human	5'-AAA AGC TGG AAG AAG CTG AAA-3'	21
Tm4	TPM4	Human	5'-AAT TAA ACT TCT GTC TGA CAA-3'	21

Table 8. Tm mouse models that recapitulate human myopathies

Gene affected (Tm isoform)	Human myopathy	Comments	Reference
TPM3 (αTm_{slow} carrying the Met9Arg mutation)	Nemaline myopathy	Human αTm_{slow} carrying the Met9Arg mutation driven by the human skeletal muscle promoter. Mice exhibit late onset nemaline myopathy.	108
Tpm1 (αTm muscle isoform carrying the Asp175Asn mutation)	Familial hypertrophic cardiomyopathy	αTm_{fast} carrying the Asp175Asn mutation driven by the cardiac specific α-myosin heavy chain promoter. The hearts exhibit a mild hypertrophic phenotype	109
Tpm1 (αTm muscle isoform carrying the Glu180Gly mutation)	Familial hypertrophic cardiomyopathy	αTm_{fast} carrying the Glu180Gly mutation driven by the cardiac specific α-myosin heavy chain promoter. The hearts develop severe concentric hypertrophy with significant ventricular fibrosis and atrial enlargement that progressively increases from 2.5 months and results in death between 4.5 and 6 months.	110
Tpm1 (αTm muscle isoform carrying the Glu54Lys mutation)	Dilated cardiomyopathy	αTm_{fast} carrying the Glu54Lys mutation driven by the cardiac specific α-myosin heavy chain promoter. Recapitulates dilated cardiomyopathy and mice exhibit changes in myofilament Ca^{2+} sensitivity.	111

mM Tris-Cl, pH 7.5, 150 mM NaCl) solution for 1 h. Primary and secondary [anti-rabbit, anti-sheep and anti-mouse Ig-conjugated horseradish peroxidise (GE Healthcare)] antibodies were incubated for 1 to 2 hr each, and 4 x 15 min washes with TTBS (TBS with 0.05% Tween 20) were carried out following each antibody incubation. The protein gel Lighting Chemiluminescence Reagent (PerkinElmer Life Sciences; Boston, MA) was used to develop the blots and blots were then exposed to Fuji X-ray Film (Kodak; Rochester, NY).

Concluding Remarks

Our current understanding of the functional significance of Tm in regulating the organizational properties and dynamic nature of the actin cytoskeleton has stemmed from the use of multiple valuable tools developed over the last few decades. This paper provides a summary of the information that is known for the four tropomyosin genes found in higher animals and an extensive list of the molecular tools and methods that have been compiled to aid in the study of these proteins. This paper has been approached from the point of view of a researcher/student who is unfamiliar with this family of proteins. We therefore, have included an overview of the evolution and regions of amino acid conservation between these genes from selected chordate species as well as information on alternative exon splicing, isoform diversity, and the ability of Tm to form distinct actin filament populations within a cellular environment. An attempt has also been made to clarify much of the misleading nomenclature (**Table 2**) and incorrect exon sequences that are frequently encountered when attempting to retrieve Tm sequences from online genomic and proteomic databases. The extensive list of Tm antibodies (**Table 3**) and fluorescently labeled Tm constructs (**Table 5**) that have been profiled for this paper, as well as the functional characterization of specific Tm isoforms using transgenic and knockout mouse models (**Table 8 and 9**), will provide valuable reference information and aid in experimental work for any newcomer to this field.

We highlight the fact that due to the high degree of evolutionary conservation between Tms in vertebrate species, the tools presented in this paper are likely to be useful to researchers working with a wide range of animal models. A field of research where the work presented in this paper would be of particular value is in MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) mass spectrometry. Fragmented protein samples which are analysed by MALDI-TOF mass spectrometry and then compared to peptide databases will often return numerous hits for Tms. Due to the high degree of similarity between the four Tm genes, determining specific Tm isoforms

Table 9. Tm mouse models generated to study the functional differences among the isoforms

Gene affected (Tm isoform)	Comment	Reference
Tpm1 (Tm3)	Rat Tm3 driven by the human β-actin promoter. The transgene causes alterations in Tm3-associated actin filaments, resulting in dystrophic features in some skeletal muscles and susceptibility to exercise-induced damaged and reduced neurite outgrowth in isolated cultured neurons.	65, 80, 90, 91
TPM3 (Tm5NM1)	Human Tm5NM1 driven by the human β-actin promoter. The transgene results in alterations in Tm5NM1-associated actin filaments that enhance neurite outgrowth in isolated cultured neurons.	16, 80, 92
Tpm3 (γTm muscle)	αTm_{slow} driven by the cardiac specific α-myosin heavy chain promoter. No morphological abnormalities in the sarcomeres of the heart. However, physiological assessment of the hearts reveals a hyper dynamic effect on systolic and diastolic performance.	112
Tpm1 (αTm muscle)	αTm_{fast} driven by the cardiac specific α-myosin heavy chain promoter. The hearts from these mice exhibit no morphological or physiological changes.	110, 113
Tpm2 (βTm muscle)	βTm driven by the cardiac specific α-myosin heavy chain promoter. No morphological or pathological alterations in cardiac morphology or sarcomeric structures are observed. However, there is an increase in the activation of the thin filament by strongly bound cross-bridges, an increase in the Ca^{2+} sensitivity and a decrease in the rightward shift of the Ca^{2+}-force relation induced by cAMP-dependent phosphorylation.	93,114, 115
(Chimericα/βTm-1)	Chimera 1: α_fTm:aa 1–257 and βTm: aa 258–284. Substitution of the carboxyl terminal end of αTm for that of βTm. Transgene driven by cardiac specific α-myosin heavy chain promoter. No morphological or pathological changes are observed in the hearts or myofibers. However, hearts exhibit functional alterations in cardiac performance with a decrease in their rates of contraction and relaxation.	116
(Chimeric α/βTm-3)	Chimera 3: replaces aa 175–190 of α_fTm with the corresponding sequence in βTm. Transgene driven by cardiac specific α-myosin heavy chain promoter. No morphological or pathological changes are observed in the hearts. However, there were decreases in the maximum rates of contraction and relaxation.	117
(Chimeric α/βTm-2)	Chimera 2: replaces aa 175–190 and 258–284 of α_fTm with the corresponding sequence in βTm. Transgene driven by cardiac specific α-myosin heavy chain promoter. Hearts exhibit abnormalities in cardiac performance, especially with decreases in their rates of contraction and relaxation.	118
Tpm3 (Deletion of all cytoskeletal products from the *Tpm3* gene)	Targeted deletion of exon 1b from the *Tpm3* gene eliminating all cytoskeletal products from this gene. Embryonic lethal.	87
Tpm3 (Deletion of Tm5NM4 and Tm5NM7)	Targeted deletion of exon 9c from the *Tpm3* gene eliminating Tm5NM4 and Tm5NM7. Histological examination of the brains from these mice reveals no gross morphological defect.	119
Tpm3 (Deletion of Tm5NM1 and Tm5NM2)	Targeted deletion of exon 9d from the *Tpm3* gene eliminating Tm5NM1 and Tm5NM2. Skeletal muscle from these mice have altered excitation-contraction coupling. Small impact on neurite outgrowth in isolated cultured neurons. Primary embryonic fibroblasts have altered motility properties.	76, 90, 92, 93
Tpm1 (Deletion of the muscle αTm isoform from the *Tpm1* gene)	Mice die between embryonic day 9.5 and 13.5	86
Tpm2 (Deletion of the muscle βTm isoform from the *Tpm1* gene)	Embryonic lethal.	84, Rajan and Wieczorek, unpublished data

from MALDI-TOF data is difficult. We strongly advise that an alignment of all the peptides generated via this methodology should be performed. The use of **Figures 2–5**, which show the amino acid sequence alignment of the Tm genes from 6 animal species, should provide the exon structure and ought to narrow down the identification of the Tm isoform(s) of interest.

There are however, a lot of questions still to be answered regarding the function of this family of proteins and one of the most intriguing is whether actin filaments consist of homo-polymers of a specific Tm isoform. Eventhough muscle Tms can form homodimers, α and β Tms prefentially form stable α/β heterodimers in native muscle. The assembly preference of the cytoskeletal Tms still remains to be established in an in vivo setting (reviewed in ref. 94). The recent development of super high-resolution fluorescent microscopy techniques (e.g., PALM, STED microscopy, etc.,),[95] may allow for a much clear discrimination of individual actin filaments populations defined by different Tm isoforms. This technology could also be used to define the molecular composition of actin filaments not only identifying the Tm isoform but potentially other actin binding/signalling proteins associating with a specific population of filaments.

Acknowledgments

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