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Alternatively spliced N-terminal exons in tropomyosin isoforms do not act as autonomous targeting signals

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ABSTRACT

Tropomyosin (Tm) polymerises head-to-tail to form a continuous polymer located in the major groove of the actin filament. Multiple Tm isoforms are generated by alternative splicing of four genes, and individual isoforms show specific localisation patterns in many cell types, and can have differing effects on the actin cytoskeleton. Fluorescently-tagged Tm isoforms and mutants were expressed in C2C12 cells to investigate the mechanisms of alternative localisation of high molecular weight (HMW) and low molecular weight (LMW) Tms. Fluorescently-tagged Tm constructs show similar localisation to endogenous Tms as observed by antibodies, with the HMW Tm3 relatively diminished at the periphery of cells compared to LMW isoforms Tm5b or Tm5NM1. Tm3 and Tm5b only differ in their N-terminal exons, but these N-terminal exons do not independently direct localisation within the cell, as chimeric mutants Tm3–Tm5NM1 and Tm5b–Tm5NM1 show an increased peripheral localisation similar to Tm5NM1. The lower abundance of Tm3 at the periphery of the cell is not a result of different protein dynamics, as Tm3 and Tm5b show similar recovery after photobleaching. The relative exclusion of Tm3 from the periphery of cells does, however, require interaction with the actin filament, as mutants with truncations at either the N-terminus or the C-terminus are unable to localise to actin stress fibres, and are present in the most peripheral regions of the cell. We conclude that it is the entire Tm molecule which is the unit of sorting, and that the alternatively spliced N-terminal exons do not act as autonomous targeting signals.

1. Introduction

Tropomyosin (Tm)[1] is an important component of most actin filaments, and has multiple roles in stabilising actin filaments and regulating their function (Gunning et al., 2005, 2008). Tm is a filamentous protein that forms coiled coil dimers, and these dimers interact head-to-tail to form a continuous polymer that runs along the major groove of the actin filament. A continuous coiled coil along the length of the protein is required for actin binding (Hitchcock-DeGregori and An, 1996). There are four mammalian Tm genes (αTm, βTm, γTm and δTm) and alternative exon splicing gives rise to three striated muscle-specific isoforms and over 40 cytoskeletal isoforms (Gunning et al., 2008). These Tm isoforms can be categorized as either high molecular weight (HMW, 284 amino acids) or low molecular weight (LMW, 248 amino acids) depending on which promoter and initial exon is used. HMW isoforms express exon 1a and either 2a or 2b, while LMW isoforms express exon 1b. Alternative splicing can also occur at exon 6, with a choice of exon 6a or 6b, and at the C-terminus with selection from exons 9a, 9b, 9c or 9d. For the most part, these exons are relatively conserved between different Tm genes, however, these genes are not redundant. Deletion of any of the αTm, βTm or γTm genes is embryonically lethal, indicating that gene products from each of these genes are required for life (Blanchard et al., 1997; Hook et al., 2004; Jagatheesan et al., 2009).

The large number of Tm isoforms allows isoform-specific regulation of the actin cytoskeleton. Tms show isoform-specific localisation patterns in many cell types (Martin and Gunning, 2008), and the specific repertoire of Tm isoforms in a cell can change throughout development and with differentiation (Schevzov and O'Neill, 2008). Tropomyosins are associated with most actin-based cellular structures, including stress fibres (Percival et al., 2000), lamellipodia (Hillberg et al., 2006), adhesion belts (Temm-Grove et al., 1998) and podosomes (McMichael et al., 2006). Tms can stabilize actin filaments by providing protection against severing by ADF (Bernstein and Bamburg, 1982; Ono and Ono, 2002) and slowing depolymerisation at the pointed end (Broschat et al., 1989). Tm

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[1] *Abbreviations used:* aa, amino acid; FRAP, fluorescent recovery after bleaching; HMW, high molecular weight; LMW, low molecular weight, Tm, tropomyosin.

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can also inhibit villin-induced actin bundling (Burgess et al., 1987) and Arp2/3-nucleated polymerisation (Blanchoin et al., 2001). Tm effects on the actin filament can be isoform-specific, with differential effects on the regulation of actin binding proteins such as gelsolin (Ishikawa et al., 1989) and formins (Wawro et al., 2007). Overexpression of different Tm isoforms can also result in altered actin filament composition and cellular morphologies, indicating differing functions (Bryce et al., 2003). Changes in Tm expression occur in cancer and other diseases (Stehn et al., 2006; Helfman et al., 2008; Das and Bajpai, 2008). In transformed cells, HMW Tms tend to be downregulated, while LMW Tm expression is maintained or increased (Stehn et al., 2006).

Although Tm is often characterized as an actin binding protein, there are few direct contacts between the Tm molecule and the actin filament. Rather, Tm "floats" above the actin filament, with the two complementarily shaped surfaces fitting neatly against each other (Holmes and Lehman, 2008). The binding strength of a single Tm molecule to actin is low, in the order of $2–5 \times 10^3\ M^{-1}$ for muscle Tms (Wegner, 1980). It is the co-operative nature of Tm provided by the end-to-end interactions between neighbouring molecules that allow Tm to associate so closely with the actin filament, leading to formation of a continuous stable Tm polymer, and eventually saturation of the actin filament with no gaps between molecules (Tobacman, 2008; Holmes and Lehman, 2008). Individual interactions between neighbouring Tm molecules are also relatively weak. These end-to-end interactions can be measured by an equilibrium constant that represents the movement of a Tm molecule from an isolated site on actin to a contiguous site, and has values between 200 and 450 M^{-1} (Wegner, 1980). End-to-end interactions are very important for co-operative binding to the actin filament, and perturbation of either end of the Tm molecule, either by mutation or by enzymatic digestion, can greatly reduce the affinity of Tm for actin *in vitro* (Cho et al., 1990; Mak and Smillie, 1981). Actin affinity can also be influenced by the specific combinations of exons in each isoform, with a choice of both exon 1 and exon 9 influencing actin affinity (Moraczewska et al., 1999; Hammell and Hitchcock-DeGregori, 1996).

In this study, we investigate the localisation of Tm isoforms in terms of their exon complement and overall sequence. We compare localisation of HMW and LMW Tm isoforms in C2C12 cells using antibodies to endogenous Tms, and show that fluorescently-tagged Tms maintain similar localisation patterns to endogenous Tms, with HMW isoforms showing lower abundance at the periphery of cells. We also investigate localisation of mutant Tms including chimeras and both N- and C-terminal deletions. We show that while the N-terminal exons of a HMW isoform Tm3 cause it to be relatively diminished at the peripheral region of cells, unlike a LMW isoform Tm5b, these N-terminal exons alone are not sufficient to regulate localisation at the periphery of cells, as a Tm3–Tm5NM1 chimera is not diminished at the periphery to the same degree. Compromising the ability of Tm3 to associate with the actin filament also relieves the peripheral diminishment, indicating that the specific localisation is actin-dependent.

2. Methods

2.1. Plasmids

Fluorescently-tagged tropomyosin construct YFP-Tm5NM1 was generated as described previously (Percival et al., 2004). YFP-Tm3 was generated by subcloning human Tm3 into pEYFP-C1 (Clontech Laboratories Inc.) using BsrG1 and BamH1 restriction sites. GFP-Tm5b was generated by subcloning rat Tm5b into pEGFP-C1 (Clontech Laboratories, Inc.) using BspEI and SalI restriction sites. Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeric mutants were generated by amplifying the N-terminal exons from human Tm3 and rat Tm5b with primers 5′-GCATGGACGAGCTGTACAAG-3′ and 5′-CTCAACCAGCTGGATCCGTC-3′. The PCR products were cloned into a YFP-Tm5NM1 plasmid with exon 1b removed using BsrG1 and BamH1 restriction sites. Tm3 N-terminal deletion mutants Tm3Δ1a and Tm3Δ1a2b were generated via PCR using primer sequences 5′-CACACGTCGACTCACATGTTGTTTAA CTC-3′ and 5′-CTCAAGCTTTTCTGGAAGATGAGCTGGTG-3′ (for Tm3Δ1a) or 5′-CTCAAGCTTTTGCTGAAGCCGACG TAG-3′ (for Tm3Δ1a2b). These mutants were cloned into a pEYFP-C1 vector using SalI and HindIII restriction sites. A Tm3 C-terminal deletion mutant lacking the final 10 amino acids of exon 9d (Tm3Δ10aa) was subcloned into pEYFP-C1 using SalI and BamHI restriction sites. All Tm fusion proteins carried the fluorescent tag at the amino terminus. The tropomyosin isoforms and mutants used in this study are shown in Fig. 1.

2.2. Cell culture and transfections

C2C12 cells were maintained at 37 °C and 5% CO_2 in a 1:1 mixture of DMEM:F12 with 15% FBS (adapted from (Cooper et al. (2004)). NIH3T3 cells were maintained at 37 °C and 5% CO_2 in DMEM with 10% FBS. Transient transfection of cells was performed with GeneJuice (Novagen) according to the manufacturers' instructions. After 24 h, cells were detached with EDTA–trypsin and replated onto coverslips for immunofluorescence or onto glass-bottomed dishes for live-cell microscopy.

2.3. Immunofluorescence

Cells on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were permeablised with cold methanol for 15 min (for Tm antibody staining) or with 0.1% Triton-X-100 in PBS for 15 min (for TRITC-phalloidin staining). Tm antibodies Tm311 (mouse, Sigma Aldrich), α/9d (sheep, Schevzov et al., 1997) and γ/9d (sheep, Percival et al., 2004) and total

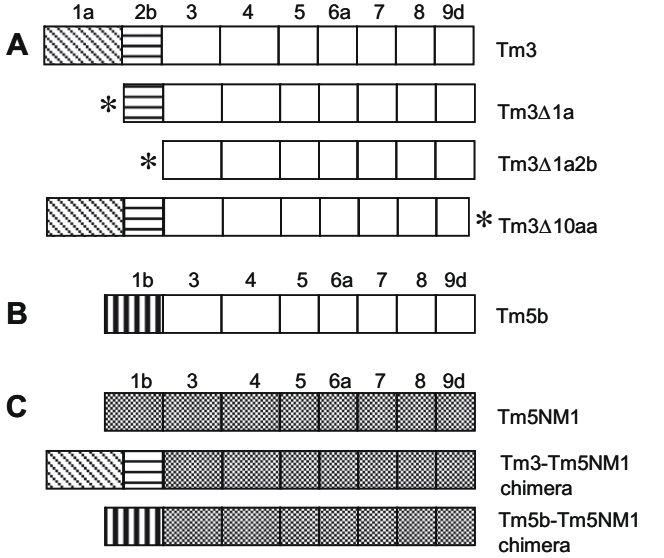

Fig. 1. Tropomyosin constructs used in this study. Tm3 and Tm5b are from the αTm gene (white boxes), while Tm5NM1 is from the γTm gene (grey boxes). Alternatively spliced N-terminal exons in the αTm gene are represented by striped boxes. HMW Tm3 contains exons 1a and 2b at the N-terminus (A). Tm3 mutants are shown with regions of deletion indicated by an asterisk. LMW Tm5b contains exon 1b at the N-terminus (B). LMW Tm5NM1 from the γTm gene also contains exon 1b (C). Tm5NM1 chimeras with the N-terminal exons of Tm3 or Tm5b are shown. All constructs are tagged with EYFP or EGFP at the N-terminus.

actin antibody C4 (a kind gift from Dr. Jim Lessard) were used. An α/9d mouse monoclonal was produced as a hybridoma using the same polypeptide, as an immunogen, as for the polyclonal. For confocal microscopy, transfected cells were counter-stained with TRITC-phalloidin (Sigma–Aldrich) to visualise actin filaments. Nuclei were stained with DAPI (Molecular Probes) and coverslips were mounted onto slides with Fluorsave™ Reagent (Calbiochem).

2.4. Microscopy

Fluorescent optical microscopy (Figs. 2–4) was performed using a Jenoptik ProgRes CF Scan digital camera with ProgRes V2.5 software mounted onto an Olympus BX50 microscope with a 40× air objective. Images were captured through U-MWV, U-MWIBA3 and U-MWIY filters. Images were captured below the saturation level of the camera. Fluorescent confocal microscopy (Fig. 6) was performed using a Leica TCS SP2 laser scanning microscope, with a 63× oil immersion objective lens. Images were captured using 488 and 543 nm laser lines. Images from different channels were merged using Photoshop CS4 (Adobe Systems Incorporated).

2.5. Image analysis

Quantification of peripheral intensity of Tm was performed using Metamorph Meta Imaging Series 6.1 (MDS Analytical Technologies Inc.). Average pixel intensity in the green channel was measured for three regions (20 × 20 pixels each) at the periphery of each cell, and divided by the average pixel intensity at three central regions of each cell adjacent to the nucleus, to normalize for different levels of protein expression between cells. At least 70 cells from two independent experiments were analysed for each experimental condition. Graphs were made and statistical analysis was performed using GraphPad Prism (GraphPad Software).

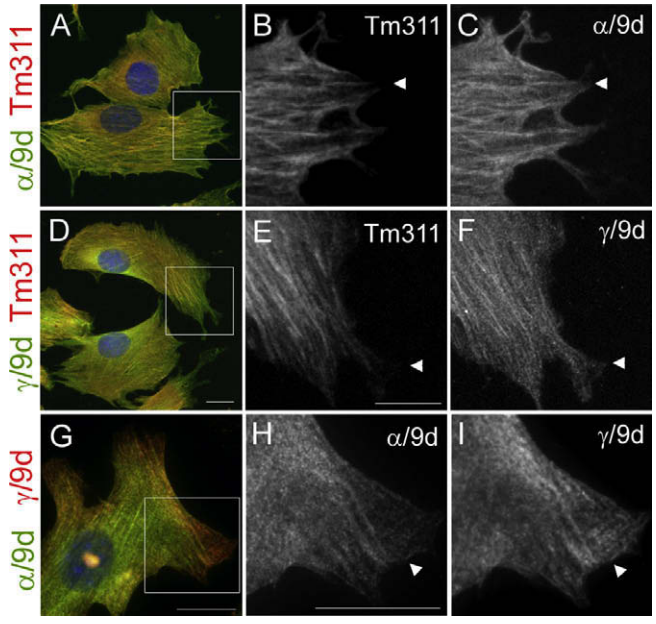

Fig. 2. Endogenous localisation of tropomyosins in C2C12 cells. Cells were double-labeled with anti-tropomyosin antibodies Tm311 and α/9d (A–C) or with Tm311 and γ/9d (D–F). Tm311 (B, E, red in merge) recognizes HMW isoforms from the αTm gene. α/9d (C, green in merge) recognizes both HMW and LMW isoforms from the αTm gene. γ/9d (F, green in merge) recognizes LMW isoforms from the γTm gene. Double-labeling with α/9d and γ/9d was also performed (G–I). Arrowheads indicate the edge of the cell. Scale bar = 20 μm.

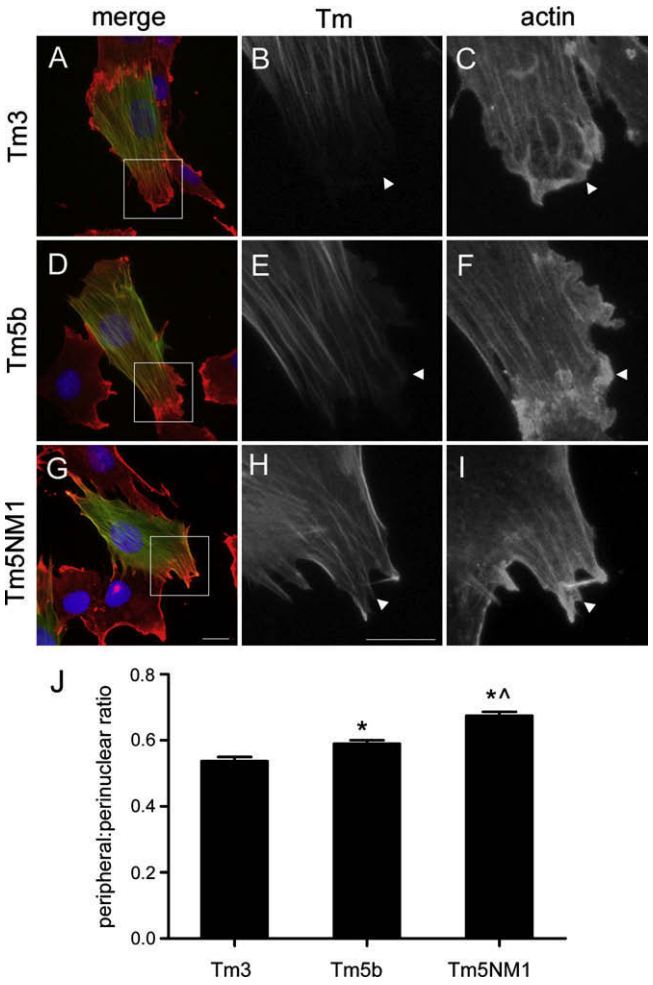

Fig. 3. Localisation of fluorescent-tagged tropomyosin isoforms in C2C12 cells. Cells were transiently transfected with fluorescently-tagged Tm3 (A–C), Tm5b (D–F), or Tm5NM1 (G–I). Middle column shows Tm (green in merge), right column shows total actin (red in merge). Arrowheads indicate the edge of the cell. Scale bar = 20 μm. The localisation of each Tm isoform at the periphery of cells was quantified by measuring pixel intensity at the periphery and normalizing to the intensity in the central region of each cell (J). Average peripheral:perinuclear intensity is shown on the *y*-axis. $^{*}p < 0.005$ (Student's *t*-test) compared to Tm3, $^{\wedge}p < 0.005$ compared to Tm5b, $n > 100$ cells/each group. Error bars indicate SEM.

2.6. Fluorescence recovery after photobleaching

FRAP analysis was performed using an Olympus FV1000 confocal inverted microscope with Olympus Fluoview FV10-ASW software. NIH3T3 cells transfected with fluorescently-tagged tropomyosin constructs were plated onto Fluorview glass-bottomed dishes (World Precision Instruments Inc.). During live-cell microscopy, cells were maintained at 37 °C with 5% CO_2 in complete media with 25 mM HEPES. Images were captured every 20 s using a 488 nm laser and 60× oil immersion lens. Three reference images were captured, then bleaching was performed in a defined region of stress fibres (ROI) for 3 s using a 405 nm SIM laser, set to 75% power. Images continued to be captured every 20 s for a total of 520 s. Fluorescent intensity in the ROI, background and total cell was measured using ImageJ (National Institutes of Health). Fluorescent intensity in the ROI was corrected for background fluorescence and overall photobleaching of the cell, and normalized to the pre-bleach intensity. For each Tm isoform 8–20 cells were analysed. The mobile fraction and half-time of recovery were

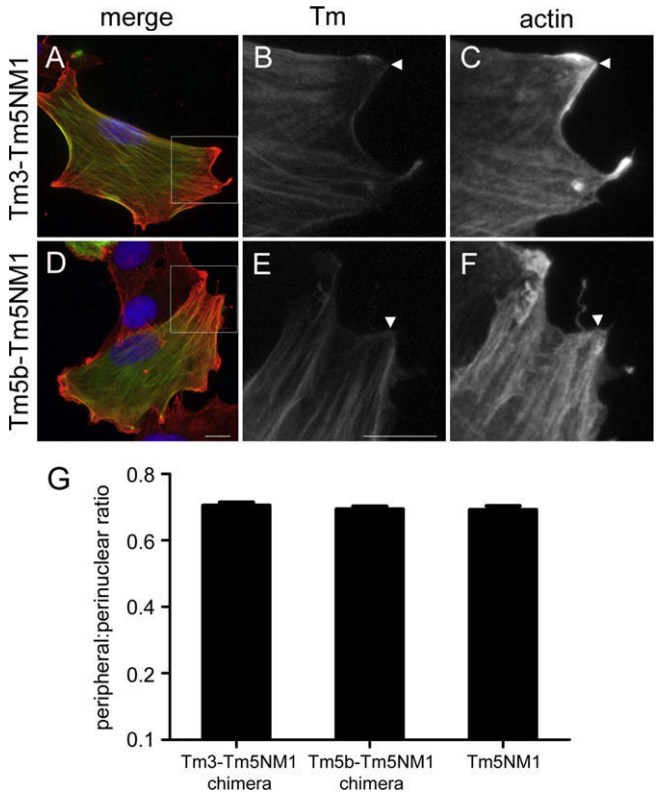

Fig. 4. Localisation of αTm–γTm chimeric mutants in C2C12 cells. Cells were transiently transfected with fluorescently-tagged chimeric mutants Tm3-Tm5NM1 (A–C) and Tm5b–Tm5NM1 (D–F). Cells were stained for total actin (C, F, red in merge). Arrowheads indicate the edge of the cell. Scale bar = 20 μm. The localisation of each Tm isoform at the periphery of cells was quantified by measuring pixel intensity at the periphery and normalizing to the intensity in the central region of each cell (G). Average peripheral:perinuclear intensity is shown on the *y*-axis. No significant differences were found by Student's *t*-test, $n > 70$ cells/each group. Error bars indicate SEM.

calculated by fitting a one-phase exponential association curve ($R = M_1(1 - \exp(-kt))$) to the data using GraphPad Prism (GraphPad Software). A one-phase curve was found to fit the data better than a two-phase curve. M_1 represents the mobile fraction, and k represents the rate constant. The half-time of recovery ($t_{1/2}$) was calculated by $\ln 2/k$. Average recovery curves for each isoform were plotted as fluorescence as a function of time.

2.7. Western blotting

Protein lysates were extracted from transfected C2C12 cells with DTT solubilisation buffer (10 mM Tris pH 7.6, 2% SDS, 2 mM DTT). Lysates were run on 12.5% SDS acrylamide gels and transferred to PVDF membranes. Blots were probed with α/9d primary antibody, and HRP-conjugated anti-sheep secondary antibody (Santa Cruz Biotechnology Inc.).

3. Results

3.1. HMW Tm isoforms are less abundant at the periphery of cells compared to LMW isoforms

C2C12 cells are a mouse myoblast cell line. In culture, the undifferentiated cells have prominent actin stress fibres, and exhibit ruffling membranes and protruding actin-rich structures at the periphery of the cells. Endogenous Tm isoforms, as observed by immunofluorescent staining, localise predominately to actin stress fibres, and also show diffuse staining in the cytoplasm of the cell (Fig. 2). Tm appears to be relatively diminished at the periphery of the cell compared to actin (not shown), consistent with previous studies in a number of different cell types (Tang and Ostap, 2001; DesMarais et al., 2002; Gupton et al., 2005; Hillberg et al., 2006). Differences in antibody staining at the periphery of the cell were observed for different Tm antibodies. Tm311 recognises HMW isoforms Tm1 from the βTm gene and Tm2 and Tm3 from the αTm gene, all of which are expressed in C2C12 cells (Schevzov et al., 1993). α/9d recognises Tm1 and both HMW (Tm2,3) and LMW (Tm5a,5b) isoforms from the αTm gene (Schevzov et al., 1997), and γ/9d recognises LMW isoforms (Tm5NM1/2) from the γTm gene (Percival et al., 2004). Co-staining of cells with Tm311 and either α/9d or γ/9d showed Tm311 had a lower intensity of staining in the peripheral regions of cells (Fig. 2B and E) compared to α/9d (Fig. 2C) or γ/9d (Fig. 2F). This is in agreement with previous observations in fibroblasts (Lin et al., 1988; Schevzov et al., 2005) where HMW isoforms are highly diminished at the peripheral regions of the cell. Similar antibody staining patterns, with lower intensity of Tm311 staining at the periphery of cells, were also seen in NIH3T3 fibroblasts (not shown). These results indicate that the HMW isoforms from the αTm gene recognized by Tm311, including Tm2 and Tm3, are relatively excluded from the periphery of these cells. LMW isoforms from the αTm gene Tm5a and/or Tm5b, recognized by α/9d but not Tm311, are not excluded from the periphery to the same degree (Fig. 2A–C). Similarly, LMW isoforms Tm5NM1/2, recognized by the γ9d antibody, are more abundant at the periphery of cells than HMW isoforms (Fig. 2D–F). Co-staining with γ/9d and an α/9d monoclonal antibody indicates that the presence of LMW isoforms from the αTm gene decreased markedly towards the periphery of the cell relative to LMW isoforms from the γTm gene (Fig. 2G–I).

3.2. Tm3 is less abundant at the periphery of cells compared to Tm5b or Tm5NM1

The anti-Tm antibodies used here recognize multiple isoforms, rather than individual isoforms. To identify the localisation patterns of specific Tm isoforms, plasmids containing a YFP or GFP fluorescent tag fused to the N-terminus of specific Tm isoforms were transfected into cells. Fluorescently-tagged Tm fusion protein have been previously studied in a number of different cell types. GFP-tagged Tm can incorporate into functionally significant actin structures in MTLn3 cells (Hillberg et al., 2006), and in myofibrils (Wang et al., 2007). In cardiomyocytes, GFP-tagged Tm not only entered into myofibrils, but the cells continued to beat and exhibit sarcomere shortening (Helfman et al., 1999), indicating that GFP-tagged Tm behaves similarly to endogenous Tm. *In vitro* studies also indicate that Tm with an 80-residue influenza virus protein extension at the N-terminus is able to bind well to actin (Hitchcock-DeGregori and Heald, 1987). Previous studies with overexpressed Tms have indicated that these Tms localise to the same compartments as endogenous Tms (Schevzov et al., 2005, 2008).

C2C12 cells were transfected with fluorescently-tagged Tm isoforms Tm3, Tm5b and Tm5NM1 (Fig. 3). Tm3 is a HMW isoform from the αTm gene and contains exons 1a and 2b at the N-terminus (see Fig. 1). Tm5b is a LMW isoform from the αTm gene and contains exon 1b at the N-terminus. Tm5NM1 is a LMW isoform from the γTm gene and has a similar exon structure to Tm5b. All isoforms studied contain exon 6a, and have exon 9d at the C-terminus. These fluorescently-tagged Tm isoforms localised predominately to actin stress fibres and showed similar patterns of localisation to the endogenous Tm isoforms at the periphery of cells. Like the Tm antibodies, all exogenous Tms studied are

present at relatively low abundance at the periphery of cells compared to actin, but different isoforms show different peripheral intensities. The HMW isoform Tm3 was lowest in abundance at the periphery of cells (Fig. 3A–C), correlating with the observed results of Tm311 antibody staining (cf. Fig. 2). The LMW Tm5b was stronger in intensity at the peripheral region of the cell than Tm3 (Fig. 3D–F), and the LMW γTm isoform Tm5NM1 showed stronger signal intensity at the periphery than both Tm3 and Tm5b (Fig. 3G–I). These differences were significant, and quantitation is shown in Fig. 3J. These results are consistent with results from the endogenous Tms in these cells (Fig. 2), and with previously published observations that show LMW isoforms including Tm5NM1 are present at higher concentrations in lamellapodia than other isoforms (Hillberg et al., 2006). Tm3 and Tm5b are identical in sequence except for the N-terminal exons, where Tm3 contains exons 1a and 2b, and Tm5b contains exon 1b (Fig. 1). This indicates that it is these N-terminal exons responsible for the different localisations of Tm3 and Tm5b. To investigate whether these N-terminal exons are independently responsible for the difference in localisation of these two isoforms, chimeric αTm-γTm mutants were made that contain the N-terminal exons of Tm3 or Tm5b fused to the C-terminus of Tm5NM1 (see Fig. 1).

3.3. Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeric mutants show similar localisation and protein dynamics to Tm5NM1

Fluorescently-tagged Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeric mutants were transfected into C2C12 cells (Fig. 4). There was no apparent difference in localisation between these two isoforms. Both localised to actin stress fibres and appeared to have relatively strong intensity at the periphery of the cells, similar to Tm5NM1. Quantitation, indicating no significant differences between the chimeric isoforms and Tm5NM1, is shown in Fig. 4G. This is in contrast to wild-type Tm3 and Tm5b which both show reduced intensity at the periphery of cells compared to Tm5NM1, with Tm3 appearing to be highly diminished at the most peripheral regions of the cell (Fig. 3A–C). These results indicate that the N-terminal exons of Tm3, exons 1a and 2b, cannot restrict the localisation of a protein with the Tm5NM1 C-terminus. The N-terminal exons of αTm alone are therefore not sufficient to direct or restrict Tm localisation; rather, the impact of the N-terminus is dependent on the rest of the protein. Exons 1a and 2b can only restrict the localisation of Tm3 in the context of a molecule with the αTm C-terminus. It is not known how much of the αTm C-terminus is required to enable the N-terminal exons to restrict location in the cell. Tm5NM1 and the Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeras show similar localisation patterns, and share the γTm C-terminus. It may be that the C-terminus of these molecules is responsible for determining their peripheral localisation.

To investigate whether the difference in peripheral localisation of Tm isoforms resulted from variation in the rate of incorporation into actin filaments by the different Tm isoforms, fluorescence recovery after photobleaching (FRAP) experiments were performed (Fig. 5). We compared the dynamics of Tm3, Tm5b, Tm5NM1 and the chimeric mutants by comparing rates of recovery after photobleaching in stress fibres. FRAP experiments were performed in NIH3T3 fibroblasts, as these cells show very prominent stress fibres in culture, and have a high transfection efficiency relative to the C2C12 cells. Analysis of the FRAP data was performed by fitting an exponential association curve to the data (see Section 2), and calculating the mobile fraction, half-time and rate constant, as shown in Table 1. All Tm isoforms studied had similar mobile fractions of 81–95%. Tm5NM1 had a significantly faster rate of association with the actin filament with a rate constant of 0.0206 compared to Tm3 (0.0082) and Tm5b (0.0079). Tm5NM1 also had a significantly faster half-time of recovery than both Tm3 and Tm5b, with a half-time of 52 s, compared to 132 and 105 s for Tm3 and Tm5b, respectively. The half-times of Tm3 and Tm5b were not found to be significantly different. The faster rate of association/dissociation for Tm5NM1 indicates that this isoform has a lower affinity for the actin filament than Tm3 and Tm5b. The Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeric mutants exhibited half-times of 69 and 72 s, respectively, more similar to Tm5NM1 than to the αTm isoforms that contributed the N-terminal exons. Although Tm5b–Tm5NM1 and Tm5b were not found to have significantly different half-times, the Tm3–Tm5NM1 chimera demonstrated a significantly faster half-time of recovery than Tm3. The N-terminal exons of Tm3 and Tm5b therefore do not appear to independently contribute to the rate of association with actin filaments. We conclude that the association rates of Tm3 and Tm5b with the actin filament cannot explain their differences in location. Tm5NM1 and the chimeric mutants, all of which have a more peripheral localisation than Tm3 or Tm5b, all show faster rates of recovery. These faster association rates with the actin filament may be a factor in the more peripheral localisation of these isoforms.

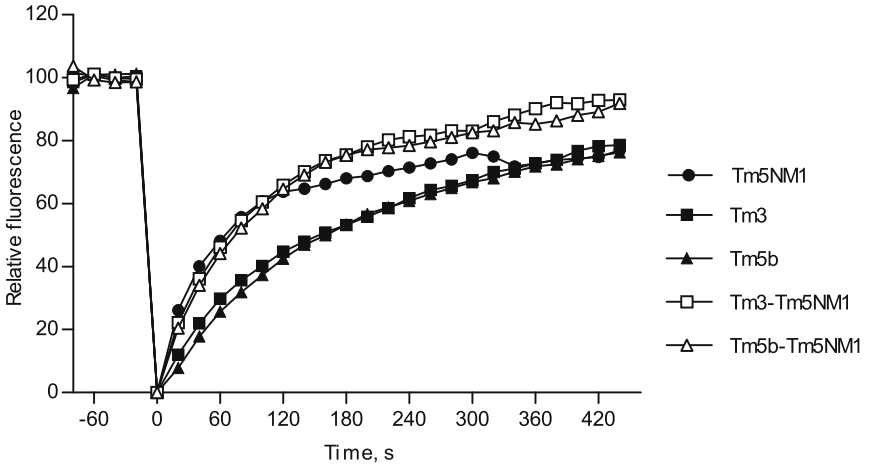

Fig. 5. Protein dynamics of Tm isoforms and chimeras in stress fibres. Fluorescent recovery after photobleaching (FRAP) experiments were performed on NIH3T3 fibroblasts transiently transfected with fluorescently-tagged Tm3, Tm5b, Tm5NM1, Tm3–Tm5NM1 and Tm5b–Tm5NM1 constructs. Average curves of 8–20 cells are plotted as recovery of fluorescence over time, with bleaching occurring at the 0 s timepoint. The mobile fraction, rate constant and $t_{1/2}$ (Table 1) were calculated as described in Section 2.

Table 1
Summary of FRAP data. After normalization (see methods), a one-phase regression curve ($R = M_1(1 - \exp(-kt))$) was fitted to the data. M_1 is the mobile fraction, k is the rate constant, and the half-time is calculated by $\ln 2/k$. Data shown are mean ± SEM, 8–20 cells were analysed for each isoform.

	Tm5NM1	Tm3	Tm5b	Tm3–Tm5NM1	Tm5b–Tm5NM1
Mobile fraction (%)	85.7 ± 4.3	90.2 ± 3.8	81.9 ± 4.8	95.0 ± 6.3	95.3 ± 9.0
$t_{1/2}$ (s)	51.8 ± 13.4*,^	131.7 ± 22.2	104.8 ± 11.6	69.4 ± 9.9*,^	71.6 ± 13.5*
k	0.0206 ± 0.0047*,^	0.0082 ± 0.0016	0.0079 ± 0.0007	0.0132 ± 0.0022	0.0132 ± 0.0030

* $p < 0.05$ compared to Tm3.
^ $p < 0.05$ compared to Tm5b by unpaired Student's t-test.

3.4. Tm3 truncation mutants do not localise to actin stress fibres

To further investigate the function of the N-terminal exons in determining Tm localisation and interaction with the actin filament, Tm3 mutants with deletions of exon 1a or both exon 1a and 2b were made. These fluorescently-tagged mutants were transfected into C2C12 cells (Fig. 6). Neither the Tm3Δ1a (Fig. 6D–F) nor the Tm3Δ1a2b (Fig. 6G–I) mutants localised to actin stress fibres. Instead these mutants were dispersed throughout the cytoplasm in a diffuse manner. This cytoplasmic staining extended all the way to the periphery of the cells, similarly to YFP alone (not shown), but unlike the wild type Tm3 (Fig. 6A–C). This indicates that deletion of the N-terminal exons has removed the peripheral exclusion of Tm3. This effect is not specific to the N-terminus, however. A Tm3 mutant with the last 10aa of the C-terminus deleted (Tm3Δ10aa, Fig. 6J–L) showed a similar pattern of localisation to the N-terminal mutants. It did not localise to stress fibres, but diffuse cytoplasmic staining extended all the way to the periphery of the cells. Similar diffuse localisation of Tm3 mutants was observed in NIH3T3 cells (not shown). Western blots of transfected C2C12 cell extracts with the α/9d antibody indicate that the YFP-tagged mutants are intact, and have not been degraded (Fig. 6M). The YFP-tagged Tms can be observed at 40–50 kDa, compared to the endogenous isoforms at 25–35 kDa. It is known that perturbation of either end of Tm can reduce actin affinity *in vitro* (Mak and Smillie, 1981; Cho et al., 1990), but this has not been extensively studied within cells. We have shown here that within cells deletion of the ends of Tm3 prevents incorporation into stress fibres of these mutants, and also removes the peripheral exclusion. The lower abundance of Tm3 at the periphery of cells therefore appears to be dependent on the ability of Tm3 to interact with actin filaments.

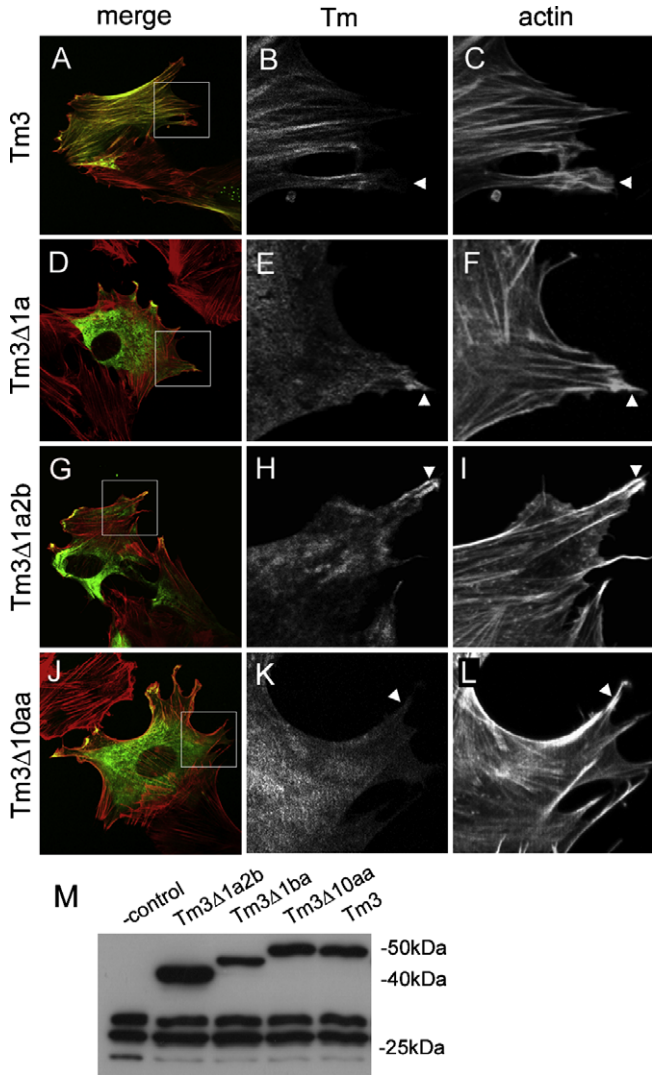

Fig. 6. Localisation of Tm3 deletion mutants in C2C12 cells. Cells were transiently transfected with YFP-tagged Tm3 (A–C), N-terminal mutants Tm3Δ1a (D–F), Tm3Δ1a2b (G–I) and C-terminal mutant Tm3Δ10aa (J–L). Cells were stained for phalloidin to visualise actin filaments (right column, red in merge). Arrowheads indicate the edge of the cell. Scale bar = 20 μm. Protein lysates from transfected cells were analysed by Western blot with the α/9d antibody (M).

4. Discussion

We have shown that tropomyosin composition differs at the cell periphery of C2C12 cells. Similar results have been demonstrated for other cell types, including NIH3T3 fibroblasts (Percival et al., 2000), MTLn3 cells (Hillberg et al., 2006), and primary fibroblasts from chicken (Lin et al., 1988) and mouse (Schevzov et al., 2005). A recurring pattern appears throughout these studies, where HMW isoforms are relatively absent from the periphery of cells. The observed sorting may be a result of both Tm sequence-specific factors that can alter the affinity of Tm for actin, and actin-filament specific factors, such as the structure of actin filaments and the presence or absence of specific actin binding proteins in a particular region of the cell. It is as yet unclear if actin filament populations at the periphery favour accumulation of LMW isoforms over HMW isoforms, or if HMW isoforms are less abundant as a result of restricted access to the periphery.

In the current study we directly compared two αTm isoforms which differ only at the N-terminus, the HMW Tm3 and the LMW Tm5b. Tm3 and Tm5b share a common C-terminus, indicating that this region is not involved in the alternate sorting of these isoforms. The difference in localisation must therefore be a result of the N-terminal exons, with exons 1a and 2b causing Tm3 to be less abundant at the most peripheral regions of the cell. These N-terminal exons cannot act in an autonomous manner, however. Chimeric mutants that have the N-terminal exons of Tm3 fused to the C-terminus of Tm5NM1 are not excluded from the periphery, but instead show very similar localisation to wild-type Tm5NM1. Likewise, a Tm5b–Tm5NM1 chimera also shows similar localisation to Tm5NM1. This indicates that the N-terminal exons of

αTm cannot act alone to direct cellular localisation, but are dependent on the context of the remainder of the protein. Tm5NM1 and the chimeric mutants that share the γTm C-terminus are the most peripherally-localised of the isoforms studied. This difference in sequence from the αTm isoforms Tm3 and Tm5b, along with the increased peripheral localisation of these γTm isoforms, indicates that the sequences of alternate genes may play an important role in localisation. We conclude that the entire protein may be the unit of sorting, rather than specific exons acting as targeting signals, for example in the manner of a mitochondrial or nuclear targeting signal.

Cytoskeletal Tms can form both homo- and hetero-dimers. HMW isoforms Tm1, 2 and 3 form homodimers only, and therefore Tm3 cannot heterodimerise with the LMW Tm5b and Tm5NM1 (Gimona et al., 1995; Gimona, 2008). This may contribute to the lower abundance of Tm3 and other HMW isoforms at the periphery compared to other LMW isoforms. LMW Tm5NM1 and Tm5b can heterodimerise with each other, as well as forming homodimers (Gimona, 2008).

Differences in protein dynamics can explain the differing localisations of some cytoskeletal proteins, such as myosins. Myosin IIA and IIB have differing localisations at the periphery of cells, and this reflects differences in the speed with which these isoforms are incorporated into structures. MyoIIA is rapidly incorporated into nascent structures at the leading edge of cells, while MyoIIB is not as quickly incorporated into these structures, and is instead more prominent at the trailing edge of cells (Kolega, 1998). The relative exclusion of Tm3 from the peripheral region of the cell does not appear to be a result of different protein dynamics, as Tm3 shows similar dynamics in FRAP experiments to Tm5b, despite differences in localisation.

Tm5NM1 has a faster rate of association/dissociation with the actin filament than both Tm3 and Tm5b. It is possible that the faster association rate may promote its preferred location at the cell periphery, as structures at the peripheral regions of the cell such as lamellapodia are very dynamic, with rapid formation and disassembly of actin filaments (Le Clainche and Carlier, 2008). Tm5NM1 has a lower affinity for actin and decreased polymerisation ability compared to HMW Tms such as Tm3 (Matsumura and Yamashiro-Matsumura, 1985; Novy et al., 1993). This may explain the faster rates of association/dissociation of this isoform with stress fibres. Tm3–Tm5NM1 and Tm5b–Tm5NM1 chimeras, which share the γTm C-terminus from exon 3 to exon 9d with Tm5NM1 also show faster rates of association with actin, indicating it may be this C-terminal region of γTm that is responsible for both the faster rates of actin association and the peripheral localisation of these isoforms.

The LMW αTm isoform Tm5a has a higher actin affinity than Tm2 (Moraczewska et al., 1999), indicating that the N-terminal exons of αTm can influence actin affinity, although no difference in fluorescent recovery was observed for Tm3 and Tm5b in the present study. Both Tm2 and Tm5a contain exon 6a, rather than exon 6b. An alternatively spliced exon 6 can influence actin affinity, with isoforms containing exon 6a having higher affinity than isoforms containing exon 6b (Hammell and Hitchcock-DeGregori, 1997). Deletion or replacement of exon 6 with a non-Tm coiled coil greatly reduces actin affinity, confirming the importance of this region of the molecule for interaction of Tm with the actin filament (Hammell and Hitchcock-DeGregori, 1997).

The lower abundance of Tm3 from the periphery of the cell cannot be explained by the rate at which it is incorporated into actin filaments, but it is clear that the ability of Tm3 to interact with actin is important for the peripheral exclusion. The localisation of the Tm3 deletion mutants demonstrates the importance of the ends of the Tm molecule in the interaction with actin. Deletion of the N-terminal exons of Tm3 inhibits the ability of the molecule to associate with actin stress fibres, and also eliminates the peripheral exclusion of Tm3. Compromising the ability of Tm3 to associate with actin filaments by deleting 10 amino acids from the C-terminal end also eliminates the peripheral exclusion. Deletion of 9–11 amino acids from the C-terminus of skeletal muscle Tm inhibits actin binding of Tm *in vitro* by inhibiting polymerisation (Mak and Smillie, 1981). Likewise, an N-terminal deletion can inhibit actin binding (Cho et al., 1990). However, in the presence of myosin S1, which can increase Tm affinity for actin, Tm with deletions at the ends will still bind actin (Moraczewska and Hitchcock-DeGregori, 2000), indicating that other cellular factors are also involved. The C2C12 and NIH3T3 cells used in this study express many actin associated proteins, as well as a range of endogenous Tm isoforms from multiple genes (Schevzov et al., 1993; Percival et al., 2000). It must be noted that *in vitro* actin binding studies performed in a minimal cell-free system containing only actin, tropomyosin and buffers may not always correlate with the effects seen in a cellular system that contains a wide variety of actin binding proteins and signaling molecules that can influence the form and composition of actin filaments. Although many *in vitro* studies have been performed, there have been few studies investigating the effect of Tm deletions within living cellular systems. Ranucci et al. (1993) investigated localisation of recombinant skeletal muscle Tm with N-terminal deletions in fibroblasts. Microinjected Tm with N-terminal deletions failed to localise to stress fibres, consistent with our results for both N-terminal and C-terminal deletions. However, unacetylated Tm, which *in vitro* has weak actin affinity, is nonetheless able to incorporate into stress fibres in cells (Ranucci et al., 1993), indicating that *in vitro* actin affinity does not always correlate with actin association within cells.

Drug studies have indicated the importance of an intact cytoskeleton for Tm localisation. Treatment of neurons with cytochalasin B leads to loss of specific localisation of Tm5NM1 and NM2 (Schevzov et al., 1997). Washout of the drug restores the normal localisation pattern. Similarly, polarised Tm distribution in epithelial cells is eliminated by cytochalasin D (Dalby-Payne et al., 2003). The sorting of Tm isoforms can therefore be considered as dependent on the assembly of specific actin structures. The differences in sorting of Tm isoforms at the periphery of the cell may be a function of the actin filaments present in the lamellipodia and other peripheral structures, which favour association with LMW isoforms over HMW isoforms. There may be competition between Tm isoforms for accumulation into these actin filaments. Actin filaments at the periphery may be associated with actin binding proteins that are mutually exclusive with Tm3, and therefore prevent accumulation of Tm3 while favouring accumulation of other isoforms such as Tm5NM1. Alternatively, rather than being actively excluded from filament populations at the periphery, Tm3 may have higher affinity for actin filaments in the central region of the cell. Tm3 would then preferentially accumulate in these more central filaments. Only when the ability of Tm3 to bind actin is impaired though mutations, is Tm3 released from these structures and able to move all the way to the periphery of cells.

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References

Bernstein, B.W., Bamburg, J.R., 1982. Tropomyosin binding to F-actin protects the F-actin from disassembly by brain actin-depolymerizing factor (ADF). Cell Motil. 2, 1–8.

Blanchard, E.M., Iizuka, K., Christe, M., Conner, D.A., Geisterfer-Lowrance, A., Schoen, F.J., Maughan, D.W., Seidman, C.E., Seidman, J.G., 1997. Targeted ablation of the murine alpha-tropomyosin gene. Circ. Res. 81, 1005–1010.

Blanchoin, L., Pollard, T.D., Hitchcock-Degregori, S.E., 2001. Inhibition of the Arp2/3 complex-nucleated actin polymerization and branch formation by tropomyosin. Curr. Biol. 11, 1300–1304.

Broschat, K.O., Weber, A., Burgess, D.R., 1989. Tropomyosin stabilizes the pointed end of actin filaments by slowing depolymerization. Biochemistry 28, 8501–8506.

Bryce, N.S., Schevzov, G., Ferguson, V., Percival, J.M., Lin, J.J., Matsumura, F., Bamburg, J.R., Jeffrey, P.L., Hardeman, E.C., Gunning, P., Weinberger, R.P., 2003. Specification of actin filament function and molecular composition by tropomyosin isoforms. Mol. Biol. Cell 14, 1002–1016.

Burgess, D.R., Broschat, K.O., Hayden, J.M., 1987. Tropomyosin distinguishes between the two actin-binding sites of villin and affects actin-binding properties of other brush border proteins. J. Cell Biol. 104, 29–40.

Cho, Y.J., Liu, J., Hitchcock-Degregori, S.E., 1990. The amino terminus of muscle tropomyosin is a major determinant for function. J. Biol. Chem. 265, 538–545.

Cooper, S.T., Maxwell, A.L., Kizana, E., Ghoddusi, M., Hardeman, E.C., Alexander, I.E., Allen, D.G., North, K.N., 2004. C2C12 co-culture on a fibroblast substratum enables sustained survival of contractile, highly differentiated myotubes with peripheral nuclei and adult fast myosin expression. Cell Motil. Cytoskeleton 58, 200–211.

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

Das, K.M., Bajpai, M., 2008. Tropomyosins in human diseases: ulcerative colitis. Adv. Exp. Med. Biol. 644, 158–167.

DesMarais, V., Ichetovkin, I., Condeelis, J., Hitchcock-Degregori, S.E., 2002. Spatial regulation of actin dynamics: a tropomyosin-free, actin-rich compartment at the leading edge. J. Cell Sci. 115, 4649–4660.

Gimona, M., 2008. Dimerization of tropomyosins. Adv. Exp. Med. Biol. 644, 73–84.

Gimona, M., Watakabe, A., Helfman, D.M., 1995. Specificity of dimer formation in tropomyosins: influence of alternatively spliced exons on homodimer and heterodimer assembly. Proc. Natl. Acad. Sci. USA 92, 9776–9780.

Gunning, P., O'Neill, G., Hardeman, E., 2008. Tropomyosin-based regulation of the actin cytoskeleton in time and space. Physiol. Rev. 88, 1–35.

Gunning, P.W., Schevzov, G., Kee, A.J., Hardeman, E.C., 2005. Tropomyosin isoforms: divining rods for actin cytoskeleton function. Trends Cell Biol. 15, 333–341.

Gupton, S.L., Anderson, K.L., Kole, T.P., Fischer, R.S., Ponti, A., Hitchcock-Degregori, S.E., Danuser, G., Fowler, V.M., Wirtz, D., Hanein, D., Waterman-Storer, C.M., 2005. Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. J. Cell Biol. 168, 619–631.

Hammell, R.L., Hitchcock-Degregori, S.E., 1996. Mapping the functional domains within the carboxyl terminus of alpha-tropomyosin encoded by the alternatively spliced ninth exon. J. Biol. Chem. 271, 4236–4242.

Hammell, R.L., Hitchcock-Degregori, S.E., 1997. The sequence of the alternatively spliced sixth exon of alpha-tropomyosin is critical for cooperative actin binding but not for interaction with troponin. J. Biol. Chem. 272, 22409–22416.

Helfman, D.M., Berthier, C., Grossman, J., Leu, M., Ehler, E., Perriard, E., Perriard, J.C., 1999. Nonmuscle tropomyosin-4 requires coexpression with other low molecular weight isoforms for binding to thin filaments in cardiomyocytes. J. Cell Sci. 112 (Pt. 3), 371–380.

Helfman, D.M., Flynn, P., Khan, P., Saeed, A., 2008. Tropomyosin as a regulator of cancer cell transformation. Adv. Exp. Med. Biol. 644, 124–131.

Hillberg, L., Zhao Rathje, L.S., Nyakern-Meazza, M., Helfand, B., Goldman, R.D., Schutt, C.E., Lindberg, U., 2006. Tropomyosins are present in lamellipodia of motile cells. Eur. J. Cell Biol. 85, 399–409.

Hitchcock-Degregori, S.E., An, Y., 1996. Integral repeats and a continuous coiled coil are required for binding of striated muscle tropomyosin to the regulated actin filament. J. Biol. Chem. 271, 3600–3603.

Hitchcock-Degregori, S.E., Heald, R.W., 1987. Altered actin and troponin binding of amino-terminal variants of chicken striated muscle alpha-tropomyosin expressed in *Escherichia coli*. J. Biol. Chem. 262, 9730–9735.

Holmes, K.C., Lehman, W., 2008. Gestalt-binding of tropomyosin to actin filaments. J. Muscle Res. Cell Motil. 29, 213–219.

Hook, J., Lemckert, F., Qin, H., Schevzov, G., Gunning, P., 2004. Gamma tropomyosin gene products are required for embryonic development. Mol. Cell. Biol. 24, 2318–2323.

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

Jagatheesan, G., Rajan, S., Wieczorek, D.F., 2009. Investigations into tropomyosin function using mouse models. J. Mol. Cell. Cardiol., in press.

Kolega, J., 1998. Cytoplasmic dynamics of myosin IIA and IIB: spatial 'sorting' of isoforms in locomoting cells. J. Cell Sci. 111 (Pt. 15), 2085–2095.

Le Clainche, C., Carlier, M.F., 2008. Regulation of actin assembly associated with protrusion and adhesion in cell migration. Physiol. Rev. 88, 489–513.

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

Mak, A.S., Smillie, L.B., 1981. Non-polymerizable tropomyosin: preparation, some properties and F-actin binding. Biochem. Biophys. Res. Commun. 101, 208–214.

Martin, C., Gunning, P., 2008. Isoform sorting of tropomyosins. Adv. Exp. Med. Biol. 644, 187–200.

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

McMichael, B.K., Kotadiya, P., Singh, T., Holliday, L.S., Lee, B.S., 2006. Tropomyosin isoforms localize to distinct microfilament populations in osteoclasts. Bone 39, 694–705.

Moraczewska, J., Hitchcock-Degregori, S.E., 2000. Independent functions for the N- and C-termini in the overlap region of tropomyosin. Biochemistry 39, 6891–6897.

Moraczewska, J., Nicholson-Flynn, K., Hitchcock-Degregori, S.E., 1999. The ends of tropomyosin are major determinants of actin affinity and myosin subfragment 1-induced binding to F-actin in the open state. Biochemistry 38, 15885–15892.

Novy, R.E., Sellers, J.R., Liu, L.F., Lin, J.J., 1993. In vitro functional characterization of bacterially expressed human fibroblast tropomyosin isoforms and their chimeric mutants. Cell Motil. Cytoskeleton 26, 248–261.

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

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

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

Ranucci, D., Yamakita, Y., Matsumura, F., Hitchcock-Degregori, S.E., 1993. Incorporation of microinjected mutant and wildtype recombinant tropomyosins into stress fibers in fibroblasts. Cell Motil. Cytoskeleton 24, 119–128.

Schevzov, G., Fath, T., Vrhovski, B., Vlahovich, N., Rajan, S., Hook, J., Joya, J.E., Lemckert, F., Puttur, F., Lin, J.J., Hardeman, E.C., Wieczorek, D.F., O'Neill, G.M., Gunning, P.W., 2008. Divergent regulation of the sarcomere and the cytoskeleton. J. Biol. Chem. 283, 275–283.

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

Schevzov, G., Lloyd, C., Hailstones, D., Gunning, P., 1993. Differential regulation of tropomyosin isoform organization and gene expression in response to altered actin gene expression. J. Cell Biol. 121, 811–821.

Schevzov, G., O'neill, G., 2008. Tropomyosin gene expression in vivo and in vitro. Adv. Exp. Med. Biol. 644, 43–59.

Schevzov, G., Vrhovski, B., Bryce, N.S., Elmir, S., Qiu, M.R., O'neill, G.M., Yang, N., Verrills, N.M., Kavallaris, M., Gunning, P.W., 2005. Tissue-specific tropomyosin isoform composition. J. Histochem. Cytochem. 53, 557–570.

Stehn, J.R., Schevzov, G., O'neill, G.M., Gunning, P.W., 2006. Specialisation of the tropomyosin composition of actin filaments provides new potential targets for chemotherapy. Curr. Cancer Drug Targets 6, 245–256.

Tang, N., Ostap, E.M., 2001. Motor domain-dependent localization of myo1b (myr-1). Curr. Biol. 11, 1131–1135.

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

Tobacman, L.S., 2008. Cooperative binding of tropomyosin to actin. Adv. Exp. Med. Biol. 644, 85–94.

Wang, J., Sanger, J.M., Kang, S., Thurston, H., Abbott, L.Z., Dube, D.K., Sanger, J.W., 2007. Ectopic expression and dynamics of TPM1alpha and TPM1kappa in myofibrils of avian myotubes. Cell Motil. Cytoskeleton 64, 767–776.

Wawro, B., Greenfield, N.J., Wear, M.A., Cooper, J.A., Higgs, H.N., Hitchcock-Degregori, S.E., 2007. Tropomyosin regulates elongation by formin at the fast-growing end of the actin filament. Biochemistry 46, 8146–8155.

Wegner, A., 1980. The interaction of alpha, alpha- and alpha, beta-tropomyosin with actin filaments. FEBS Lett. 119, 245–248.