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Distinct sites in tropomyosin specify shared and isoform-specific regulation of myosins II and V

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Abstract

Muscle contraction, cytokinesis, cellular movement, and intracellular transport depend on regulated actin-myosin interaction. Most actin filaments bind one or more isoform of tropomyosin, a coiled-coil protein that stabilizes the filaments and regulates interactions with other actin-binding proteins, including myosin. Isoform-specific allosteric regulation of muscle myosin II by actin-tropomyosin is well-established while that of processive myosins, such as myosin V, which transport organelles and macromolecules in the cell periphery, is less certain. Is the regulation by tropomyosin a universal mechanism, the consequence of the conserved periodic structures of tropomyosin, or is it the result of specialized interactions between particular isoforms of myosin and tropomyosin? Here we show that striated muscle tropomyosin, Tpm1.1, inhibits fast skeletal muscle myosin II but not myosin Va. The non-muscle tropomyosin, Tpm3.1, in contrast, activates both myosins. To decipher the molecular basis of these opposing regulatory effects, we introduced mutations at conserved surface residues within the six periodic repeats (periods) of Tpm3.1, in positions homologous or analogous to those important for regulation of skeletal muscle myosin by Tpm1.1. We identified conserved residues in the internal periods of both tropomyosin isoforms that are important for the function of myosin Va and striated myosin II. Conserved residues in the internal and C-terminal periods that correspond to Tpm3.1-specific exons inhibit myosin Va but not myosin II function. These results suggest that tropomyosins may directly impact myosin function through both general and isoform-specific mechanisms that identify actin tracks for the recruitment and function of particular myosins.

Keywords

actin cytoskeleton; actomyosin regulation; coiled coil; intracellular transport; muscle contraction

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Introduction

The eukaryotic cytoskeleton defines and maintains the shape and structure of cells, enables dynamic movement and cell division, and creates a "highway" system for molecules and organelles to reach their destinations. Actin filaments comprise a major component of the cytoskeleton, one function of which is to interact with the mechanochemical enzyme, myosin. Actin-myosin interaction produces the force required for muscle contraction, cellular movement, and cell division, as well as for the transport of subcellular cargos such as organelles and macromolecular complexes in eukaryotes. Whereas a few isoforms of actin form the tracks on which myosins move, there are many classes of myosin and actin binding proteins that modify actin filament assembly and function. One actin-binding protein is tropomyosin, a near-universal allosteric regulator of the actin filament [Gunning et al., 2015; Khaitlina, 2015; Perry, 2001; Wang and Coluccio, 2010].

Tropomyosins (Tpm) are a family of two-chained α -helical coiled-coil proteins that polymerize along the length of the actin filament, thus stabilizing it and conferring cooperativity in an isoform-specific manner. Actin binding and myosin regulation are conserved tropomyosin functions. Tropomyosins bind to and regulate the actin filament component of the cytoskeleton and its interaction with other proteins including myosin [Gateva et al., 2017; Hitchcock-DeGregori and Barua, 2017; Robaszkiewicz et al., 2016; Nevzorov and Levitsky, 2011; Colpan et al., 2017; Sui et al., 2017]. In doing so, tropomyosins specify actin tracks within cells for the localization and function of myosins and other actin binding proteins that respond to changes in cell state such as oncogenic transformation and activation of muscle contraction by Ca^{2+} [Gunning and Hardeman, 2017; Manstein and Mulvihill, 2016; Squire et al., 2017]. As we appreciate the diverse cellular functions of myosins and how they are regulated as ultimate targets in signaling cascades [Heissler and Sellers, 2016], our objective here is to address the specificity of regulation of myosin at the level of the actin filament.

Tropomyosin is best known for its role in thin (actin) filament regulation in striated muscle where regulation of contraction is allosteric, via troponin-tropomyosin on actin and binding of myosin crossbridges to actin [Gordon et al., 2000; Bremel and Weber, 1972; Geeves et al., 2011; Lehrer and Geeves, 1998; Tobacman and Butters, 2000; Karpicheva et al., 2017; Shchepkin et al., 2017; Eisenberg and Weihing, 1970]. Tropomyosin increases the affinity of myosin for actin and vice versa in the strong binding state of the actin filament [Eaton, 1976; Moraczewska et al., 1999]. Comparative studies of regulation of myosins by tropomyosin showed that both proteins influence the state of the actin filament such that tropomyosin can activate or inhibit [Lehrer and Morris, 1984; Miyata and Chacko, 1986; Williams et al., 1984]. More recent work extends this concept to non-muscle myosins and tropomyosins [Clayton et al., 2014; Fanning et al., 1994; Hodges et al., 2012; Barua et al., 2014; Skolnick et al., 2016; Pathan-Chhatbar et al., 2018]. In mammals, four tropomyosin genes encode more than forty isoforms [Geeves et al., 2015]. There are two classes: high molecular weight (HMW) isoforms (~284 residues) have seven periodic repeats (periods) that span seven actin subunits in the filament, and low molecular weight (LMW) isoforms (~247 residues) have six periods that span six actins. A particular cell type may express multiple HMW and LMW isoforms, often with specific but not exclusive localizations and functions [Gunning et al.,

2015; Wang and Coluccio, 2010]. Here we seek to understand how the myosin motor is regulated at a molecular level by determining which conserved residues in the tropomyosin sequence encode the information to regulate myosin, and if myosin regulation by tropomyosin is specific to the myosin isoform, the tropomyosin isoform, or both.

The myosin family is larger and more diverse than the tropomyosin family. It is thought that all animal and fungal myosins, with the exception of the myosin I class, move on tropomyosin-containing actin filaments [Clayton et al., 2015; Clayton et al., 2010; Fanning et al., 1994; Tang and Ostap, 2001; Brayford et al., 2016]. We have studied two of the best known here. Skeletal muscle myosin II is a non-processive, sarcomeric myosin that forms bipolar filaments and drives muscle contraction in response to Ca^{2+} binding to troponin [Gordon et al., 2000]. Mammalian myosin Va is a cytoplasmic, non-filamentous, processive motor that takes multiple steps on actin without dissociating. It is a transporter for vesicles, organelles, and macromolecules in the cell periphery [Hammer and Sellers, 2011; Hammer and Wagner, 2013; Desnos et al., 2007]. Tropomyosin is essential for some yeast class V myosins to move processively on actin [Clayton et al., 2014; Hodges et al., 2012; Tang et al., 2016]. In the case of mammalian myosin Va, cytoplasmic tropomyosins act to promote or exclude interaction of myosin Va with actin, depending on the isoform [Skolnick et al., 2016].

Our strategy to address the question of how tropomyosins are designed to regulate myosins was to compare myosin II and myosin Va function in the presence of two tropomyosin isoforms that differ in several ways: the gene encoding them, the length, the selection of alternatively-expressed exons, and their known functions. Striated muscle α -tropomyosin (Tpm1.1) is a HMW isoform expressed in striated and cardiac muscles that binds troponin and regulates muscle contraction. It has seven periodic repeats that contain sets of residues for actin binding that alternate with sets that regulate skeletal muscle myosin [Barua et al., 2013; Barua et al., 2012]. Here we test if those same sets of residues regulate myosin Va. Further, we compare how a widely-expressed LMW non-muscle tropomyosin, Tpm3.1 (Tm5NM1), carrying analogous sets of mutations, functions in regulation of myosin Va and skeletal muscle myosin II. Myosin Va and Tpm3.1 are widely-expressed in cells and are implicated in disease states including metastatic cancer, melanoma and type 2 diabetes, though we do not yet know if they function together in cells [Bonello et al., 2016; Alves et al., 2013; Chen et al., 2012; Kee et al., 2015]. The myosin and tropomyosin pairs were selected such that they form both physiologically-relevant (striated muscle myosin II/Tpm1.1, myoVa/Tpm3.1) and non-physiologically-relevant (striated muscle myosin II/Tpm3.1, myoVa/Tpm1.1) pairs. The results of our experiments lend support to the hypothesis that myosins and tropomyosins have mutual roles in local specialization of the actin cytoskeleton and its myosin-dependent functions. The ability of a tropomyosin to activate or inhibit a myosin depends on both the tropomyosin and the myosin. Mutation of conserved residues in some periods affects myosin function irrespective of the tropomyosin or myosin type suggesting that they may be isoform independent and possibly universal regulators of myosin function. These regions are distinct from conserved residues in other periods that affect the regulation of myosin Va by Tpm3.1 only and may contribute to the specification of tracks for intracellular transport.

Results

We chose Tpm1.1 and Tpm3.1 because they are the most different in structure and function amongst the vertebrate tropomyosins that have been studied. In the ensemble motility assay, Tpm1.1 inhibits skeletal muscle myosin and has no effect on a non-muscle filamentous myosin IIA while Tpm3.1 activates both myosins [Barua et al., 2014]. Tpm3.1 activates myosin Va in a single molecule processivity assay, while other non-muscle tropomyosin isoforms inhibit; Tpm1.1 has not been tested with myosin Va [Sckolnick et al., 2016].

Tpm1.1 is a striated muscle tropomyosin encoded by the *TPM1* gene; Tpm3.1 is a non-muscle (cytoskeletal) tropomyosin, encoded by the *TPM3* gene [Geeves et al., 2015]. Figure 1A compares the exon organization of the two isoforms. Figure 1B shows the sequences, aligned by the periodic repeats. The sequences are divided into seven (Tpm1.1) and six (Tpm3.1) periods according to the Phillips analysis (P1–P7) [Phillips, 1986]. Each period corresponds to the length of one actin subunit in the actin filament. The two isoforms use alternative promoters, resulting in expression of different initial exons. Tpm1.1 expresses exon 1a and exon 2b whereas Tpm3.1 expresses exon 1b and lacks a second exon that corresponds to period 2 (P2) in Tpm1.1 (Figure 1B). The two isoforms express alternative forms of exon 6, which encodes P5 and exon 9, which encodes P7.

Mutational Strategy

Our initial focus was to learn if the residues in the second halves of the periodic repeats of striated muscle tropomyosin (Tpm1.1) that are important for the allosteric regulation of skeletal muscle myosin have a comparable function in a non-muscle tropomyosin (Tpm3.1). We extended our study to include the P1 and P7 of both tropomyosins.

In a coiled-coil protein the surface residues are the most available to bind to other proteins. The first halves of the periodic repeats of tropomyosin contain an evolutionarily-conserved motif of surface acidic and basic residues that are important for binding actin [Barua et al., 2013; Barua et al., 2011], and are proposed to bind actin in models of actin-tropomyosin [Li et al., 2011]. In a previous study, we mutated the surface *b*, *c*, *f* residues of Tpm1.1 in the second halves of periods P2–P6 that are evolutionarily-conserved in animal tropomyosins (Figure 1B). We found that sites in the second halves of periods P3–P6 are the most important for skeletal muscle actomyosin regulation (Figure 2A) [Barua et al., 2012]. Here we mutated homologous conserved residues in periods P3–P6 of Tpm3.1 (Figure 1B). Tpm1.1 and Tpm3.1 sequences in the second half of periods P3–P6 are highly conserved, even though the second half of P5 is encoded by alternate sixth exons (Figure 1A). Exons 6a of Tpm3.1 and 6b of Tpm1.1 have 68% sequence similarity, if we consider K/R, D/E, N/Q and M/L/V/I as equivalent. The mutated residues in Tpm3.1-P5 are at the same positions as those in Tpm1.1-P5.

The Tpm1.1 and Tpm3.1 sequences differ the most in the second halves of P1 and P7 that are encoded by exons 1a or 1b and 9a or 9d (50% and 33% sequence similarity, respectively, Figure 1B). We mutated surface residues in the second halves of P1 and P7 that are conserved within each alternate exon and are at the same position in the alignment in both tropomyosins. For example, we mutated Lys37 in Tpm1.1-P1, conserved in exon 1a, which

aligns with a conserved Glu in exon 1b, mutated in Tpm3.1-P1. All residues were mutated to Ala, except for Leu to Ser. The wildtype and mutant tropomyosins were expressed and purified from *E. coli* and the N termini are unacetylated. Tpm1.1 has an Ala-Ser extension at the N terminus to increase actin affinity [Monteiro et al., 1994]. Unacetylated Tpm3.1 binds well to actin [Barua et al., 2014].

The recombinant tropomyosins were analyzed for actin affinity, thermal stability, and mechanochemical function with two myosins, tissue-purified chicken skeletal muscle myosin II and recombinant murine myosin Va-HMM. Cosedimentation assays and thermal stability measurements of the tropomyosin mutants ensured that the effects on actomyosin regulation are not primarily related to actin affinity or stability. Actin affinities were typically within ~2-fold of wildtype except Tpm3.1-P7, which had a 4-fold lower actin affinity (Table S1, Figure S1). All functional assays were carried out under conditions where tropomyosin saturated the actin filaments. The thermal stability of the tropomyosin mutants was determined using circular dichroism spectroscopy (Table S1, Figure S1). The melting temperature (T_M) of the mutants was within 2 °C of WT.

Regulation of Skeletal Muscle Myosin II by Tpm1.1 and Tpm3.1 Mutants

We determined the velocity of actin-tropomyosin filaments with skeletal myosin II using an ensemble motility assay that measures the speed of rhodamine phalloidin-labeled actin filaments sliding along a myosin-coated surface (Figure 2A, B, Table 1). Tpm1.1 inhibited sliding velocity by ~40% and Tpm3.1 activated velocity by ~60% relative to actin alone, confirming published work [Barua et al., 2014].¹ The tropomyosin mutants had a neutral or inhibitory effect compared to wildtype with a similar pattern for both Tpm1.1 and Tpm3.1, except for P5. The P3 and P6 mutants showed the greatest inhibition: ~60–80% relative to WT Tpm1.1 [Barua et al., 2012] ($p < 0.001$) and ~50–60% relative to WT Tpm3.1 ($p < 0.001$). The velocities with Tpm3.1-P3 and P6 mutants were also 40% and 25% slower than bare actin. The P4 and P5 mutants of Tpm1.1 inhibited filament velocity by ~30–50% [Barua et al., 2012] ($p < 0.1$, $p < 0.01$, respectively). The effect of Tpm3.1-P4 mutant was slightly less than Tpm1.1-P4 (20%) ($p < 0.01$) and that of Tpm3.1-P5 was negligible. Mutations in conserved residues of P1 and P7 had almost no effect on filament velocities for either tropomyosin, despite being encoded by alternate exons in their respective genes. The results for Tpm1.1-P3–P6 are included from Barua *et al.*, 2012 [Barua et al., 2012] to enable comparison to Tpm1.1-P1 and P7 as well as the Tpm3.1 mutants.

The results show that mutations at homologous positions in Tpm1.1 and Tpm3.1 have period-specific effects on the regulation of skeletal myosin II (Figure 2A,B). While Tpm1.1 is an isoform that naturally regulates skeletal myosin, Tpm3.1 is not. The P3, P4 and P6 mutations have similar effects in both Tpm1.1 and Tpm3.1. The results suggest that myosin regulation by these regions may be conserved across tropomyosins. The greatest departure

¹Our results with skeletal myosin II differ from those of Clayton et al., 2015 who reported that Tpm3.1 inhibits skeletal myosin II in a gliding assay. After extensive discussion and testing of each other's tropomyosin preparations we concluded the difference is due to the preparation of the gliding surface. In our assays we apply non-filamentous myosin (in high salt buffer) to the nitrocellulose surface via an anti-S2 monoclonal antibody whereas in the Clayton et al. paper, skeletal muscle myosin in the filamentous state (bipolar filaments) was applied directly to the nitrocellulose surface. Actin gliding speeds on bipolar myosin filaments were predictably slower than on monomeric myosin.

from the overall pattern is with the P5 mutations. Mutations in P5 in Tpm1.1, but not Tpm3.1, affect myosin II. The mutated regions of P5 are encoded by alternate forms of exon 6 (6a in Tpm3.1, 6b in Tpm1.1), and different genes.

Regulation of Myosin Va by Tpm1.1 and Tpm3.1 Mutants

In cells, Tpm3.1 is localized to cytoplasmic actin structures, including those involved in intracellular transport that require non-muscle myosins such as myosin Va [Gunning et al., 2015]. Recent work showed that, *in vitro*, myosin Va-HMM (a constitutively-active truncated form of myosin Va, hereafter referred to as myoVa), prefers Tpm3.1-actin over bare actin for movement [Sckolnick et al., 2016]. To understand how mutations in conserved residues of periodic repeats of tropomyosin affect a non-muscle myosin, we characterized the movement of single molecules of myoVa labeled with Q-dots along surface-adhered rhodamine-phalloidin labeled actin filaments with wildtype or mutant Tpm1.1 or Tpm3.1. We quantified speeds, run lengths, and run frequency of single myoVa molecules.

Run frequency, measured as the number of motors moving per unit time and actin length, increased ~50% with Tpm3.1-WT, as previously reported [Sckolnick et al., 2016], but Tpm1.1-WT had no effect (Figure 2C, D, Table 2). Run frequency is related to the affinity of the motor for the actin filament. The sensitivity of myosin run frequency to tropomyosin mutations suggests that tropomyosin may regulate the initial engagement of myosin with the track, i.e. affinity.

Considering that Tpm3.1 stimulated both skeletal myosin II and myoVa, one might have expected the pattern of the effects of the mutants on the two myosins to be similar. However, that was not the case. Of particular interest is that Tpm3.1-P5 and P7 reduced run frequency of myoVa by ~50% or more ($p < 0.001$), whereas skeletal myosin II was insensitive to mutations in Tpm3.1-P5 and mutations in P7 in both Tpm3.1 and Tpm1.1 (compare Figure 2A, B). However, the Tpm3.1-P1, P3, P4 and P6 mutants show a similar pattern of inhibition with both myosins in these different functional assays: P1 had virtually no effect, P4 had an intermediate effect ($p < 0.01$), and P3 and P6 reduced run frequency to less than with actin alone (~60% inhibition compared to WT Tpm3.1 ($p < 0.001$) (compare Figure 2B, D). In contrast, the pattern of the effect of Tpm1.1 mutations on myosin Va was generally similar to that with skeletal myosin II even though Tpm1.1 had no effect on myoVa run frequency: P1 and P7 had no effect, P3 and P4 had intermediate effects (~25% inhibition, $p < 0.1$), and P5 and P6 had the greatest effect (>70% inhibition, $p < 0.001$) relative to WT Tpm1.1 (Compare Figure 2A, C). Together the results provide evidence of common (if not universal) and exon-specific roles for tropomyosin in regulation of myosin function that we will discuss below.

In contrast to the effects of the WT tropomyosins on run frequency, run length and speed were unchanged (Table 2). The tropomyosin mutations also did not significantly affect speed or run length relative to WT (Table 2). These data suggest that myoVa stepping rate and dissociation rates are largely insensitive to the mutations.

Effect of Tpm3.1 on the product dissociation steps of the ATP hydrolysis mechanism of actomyosin II and Va

To relate the inhibitory and activating effects of Tpm1.1 and Tpm3.1 that we observed, we analyzed the product dissociation steps of striated muscle myosin II and myoVa in the presence of actin-tropomyosin. We previously reported that the inhibition of filament velocity by Tpm1.1 is associated with inhibition of the rate of product release following ATP hydrolysis by skeletal myosin II-S1 [Barua et al., 2012], consistent with the inhibition of ensemble motility assay results (Figure 2A and previously published work [Fraser and Marston, 1995; VanBuren et al., 1999]). Here, we used stopped-flow fluorescence to determine if Tpm3.1 affects either phosphate (Pi) or ADP dissociation rates from skeletal myosin II-S1 or myosin Va-S1 actomyosin-ADP-Pi. These steps of the hydrolysis mechanism influence the motile properties of actomyosin motors: the ADP dissociation rate generally affects velocity [De La Cruz et al., 1999; Siemankowski et al., 1985] and the Pi dissociation rate affects run length in processive myosins such as myosin Va [Forgacs et al., 2009].

Dissociation of Pi and ADP from actomyosin-ADP-Pi are ordered ($\text{AM-ADP-Pi} \rightarrow \text{AM-ADP} + \text{Pi} \rightarrow \text{AM} + \text{ADP} + \text{Pi}$). For skeletal myosin II-S1, ADP dissociation from AM-ADP-Pi is limited by the slower dissociation of Pi. The fluorescence of deoxymantADP (mdADP) decreases upon dissociation and provides a signal to measure the kinetics of Pi dissociation from AMII-mdADP-Pi [Heeley et al., 2006]. In Figure 3A, myosin II-S1 was mixed with mdATP to produce MII-mdATP and MII-mdADP-Pi, which was then mixed with actin or actin-Tpm3.1. The observed rate increases with the concentration of F-actin. Pi dissociation limits the maximum rate of mdADP dissociation from AMII-mdADP-Pi to $89 \pm 10 \text{ s}^{-1}$. The rate is not affected by Tpm3.1, nor is a second slower rate of $\sim 9 \text{ s}^{-1}$ that originates from attached hydrolysis ($\text{A-MII-mdATP} \rightarrow \text{AMII-mdADP-Pi}$). The ~ 4 -fold faster rate of mdADP dissociation from AMII-mdADP, $\sim 400 \pm 50 \text{ s}^{-1}$, is also unaffected by Tpm3.1 bound to the F-actin (Figure 3B). The results with Tpm3.1 differ from Tpm1.1, which produced a ~ 3 -fold decrease in the rate of product dissociation [Barua et al., 2012].

For myosin Va-S1, Pi dissociation also precedes ADP dissociation, but the ADP dissociation is rate limiting. The rate of Pi dissociation from A-MVa-ADP-Pi was measured by double mixing stopped flow fluorescence, where Pi dissociation was measured directly using fluorescent phosphate binding protein. The rate of Pi dissociation at saturating actin is reduced $\sim 40\%$ in the presence of Tpm3.1 bound to actin (Figure 3C). Slower Pi dissociation would decrease the probability of lead head attachment occurring before trailing head dissociation, resulting in a shorter run for a processively walking myoVa [Forgacs et al., 2009]. However, we did not observe any change in run length of myoVa in the presence of Tpm3.1 in the single molecule processivity assays described above. Tpm3.1 has no significant effect on the observed rate constant of ADP dissociation from A-MVa-ADP (Figure 3D). This is consistent with our observation of no effect of Tpm3.1 on the run speeds of myoVa.

Discussion

It is well-established that regulation of myosin by tropomyosin depends on both the myosin and tropomyosin isoform [Hitchcock-DeGregori and Barua, 2017]. Here we investigated whether mutation of specific conserved residues in different tropomyosin isoforms has the same or different impact on the function of a class II myosin and a class V myosin. Our results suggest that the mutated residues in the second halves of the highly-conserved periods of tropomyosin, P3, P4 and P6, regulate myosin function through a common mechanism. In contrast, the mutated residues in P5 and P7, encoded by alternate exons 6b or 6a and 9a or 9d, respectively, in Tpm1.1 and Tpm3.1 differentially influence the functions of the two myosins (Figure 4).

Tpm1.1 and Tpm3.1 have different effects on skeletal muscle myosin II and myoVa. Tpm1.1 inhibits the gliding speed of actin filaments on myosin II in an ensemble motility assay, but does not affect run frequency of myoVa in a single molecule processivity assay. Tpm3.1 activates skeletal muscle myosin II in an ensemble motility assay and enhances run frequency of myoVa in a single molecule processivity assay. Speeds from the processivity assay, unaffected by either tropomyosin isoform, are not directly comparable to speeds measured in an ensemble motility assay. A single molecule assay determines speeds of double-headed molecular motors that are gated by intramolecular strain between the heads, while speeds in an ensemble motility assay are the result of a combination of power strokes from many individual, independent myosins. A change in the affinity of myosin II for the track will essentially manifest as a decrease/ increase in the effective number of motors on the surface that can engage, thus resulting in inhibition/ activation of ensemble motility speeds. In contrast, an increase in affinity for a high duty ratio motor such as myosin Va will manifest as an increase in run frequency in a single molecule assay, as seen for Tpm3.1. Tpm1.1 and Tpm3.1 may have preferred binding sites on the actin filament in the absence of myosin, with Tpm1.1 partially occupying the myosin binding site on the outer domain of the actin [Lehman et al., 2000], thus inhibiting binding of myosin to actin, whereas Tpm3.1 may be on the inner domain that does not interfere with and may enhance the binding of myosin to actin in the strong binding state, likely through direct interaction with myosin (discussed in the next section). We suggest that there is a potential for interactions between tropomyosin and myosin in the open state of actin, where tropomyosin may bind to either myosin-ADP or rigor myosin or both. We are unable to determine the state of the myosin based on the assays we carried out.

We observed no changes in the rates of Pi or ADP dissociation of both myosins in the presence of Tpm3.1, except a 40% decrease of the rate of Pi dissociation of myosin Va in the presence of Tpm3.1. We anticipated that the increased filament speed of actin-Tpm3.1 on myosin II would be correlated with an increased rate of ADP dissociation, but this was not the case. We do not have a particular explanation for the apparent discrepancy, but note that the two assays used different forms of myosin II (full-length myosin vs subfragment 1) and were carried out at different salt concentrations selected to optimize the particular assay.

Common mechanism

The sequences of P3, P4 and P6 are nearly identical in Tpm1.1 and Tpm3.1. They are encoded by constitutively-expressed exons (exons 3, 4, 5, 7, 8) in different genes (Figure 1). The qualitatively similar effect (Figure 4) of the mutations in P3, P4, and P6 on two myosins that differ in their evolution and cellular functions suggests that these two tropomyosins regulate the two myosins by similar mechanisms. To relate the inhibition of myosin function by tropomyosin mutants to a structural model, we constructed molecular models of tropomyosin-actin-myosin with the rigor myosin Va-S1 [Coureux et al., 2003] or striated myosin II-S1 [Winkelmann et al., 2015] crystal structures (Figure 5, Materials and Methods). We docked a 7 Å-resolution crystal structure of Tpm1.1 [Whitby and Phillips, 2000] on a 8 Å rigor actin-tropomyosin-myosin S1 cryo-EM structure [Behrmann et al., 2012] and modified its position to optimize potential electrostatic interactions of the residues mutated in two internal periods that had the largest effects on regulation, P3 and P6, with charged residues on myosin Va or myosin II (Figure 5) [Barua et al., 2012]. Doing so resulted in an axial shift of ~ 37 Å of the tropomyosin but maintained the azimuthal position of tropomyosin on actin. We note that there is no structural information to define the axial position of tropomyosin on actin.

In the models, the conserved residues mutated in P3 and P6 can interact with oppositely charged residues in and around loop 4 in the upper 50K domain of myosin II and Va (details shown in Figure 5). Tpm1.1 and Tpm3.1 are likely to have similar interactions with the two myosins. We propose the residues mutated in P3 and P6 may stabilize the open/activated state through weak but specific electrostatic interactions with myosin, thus shifting the equilibrium of actin-tropomyosin filaments to the open state. When the proposed interactions with myosin are lost in the tropomyosin mutants, the blocked/closed to open transition will not be favored, leading to rates below those of actin-WT tropomyosin or actin alone. In support of the structural models, crosslinking and binding studies have shown direct interaction of tropomyosin with myosin (Barua and Hitchcock-DeGregori, unpublished results) [Golitsina and Lehrer, 1999; Merkel et al., 1989; Tao and Lamkin, 1984].

Tropomyosin periods encoded by alternatively-expressed exons can differentially affect myosin function

Of particular significance is our finding that mutations in P5 and P7 of Tpm1.1 and Tpm3.1 have different effects on the two myosins we studied. Alternatively-spliced exons 6 and 9 encode P5 and P7 (Figure 1), respectively, regions that we propose have isoform-specific roles in regulation of myosin function.

The most variable regions in tropomyosins are those encoded by the alternate 5' and 3' coding exons, 1a/1b and 9a/9b/9c/9d, the sequences that form the junction between tropomyosin molecules along the actin filament. Even though the 1a/9a, 1b/9d and 1a/9d junctional complexes have similar structures [Frye et al., 2010; Greenfield et al., 2006; Greenfield et al., 2009], they differ in functionality. Exon 9a encodes a sequence expressed in striated muscles that binds troponin T, a regulatory protein found only in cardiac and skeletal striated muscles [Cho and Hitchcock-DeGregori, 1991; Mak and Smillie, 1981;

Palm et al., 2003]. The first part of exon 9a, the site of three of the four mutations in P7, is essential for binding troponin T [Hammell and Hitchcock-DeGregori, 1996; Moraczewska and Hitchcock-DeGregori, 2000].

Our results indicate that the region of exon 9 mutated in the P7 mutants has isoform specific functions: troponin T binding in Tpm1.1 (exon 9a) and myosin regulation in Tpm3.1 (exon 9d). The Tpm1.1-P7 mutant has no effect on skeletal myosin or myoVa function. In contrast, the Tpm3.1-P7 mutant inhibits myoVa while having no effect on skeletal muscle myosin (Figure 2, Figure 4). We suggest *TPM3* exon 9d encodes a region that is specialized to regulate myosin Va, and perhaps other non-striated muscle myosins. The exon 9-encoded sequences are variable among the alternate forms and the four genes. Additional functions remain to be discovered.

Mutations in P5 of Tpm1.1 and Tpm3.1 also have different effects on the two myosins (Figure 2, Figure 4). Tpm1.1-P5, in which P5 is encoded by exon 6b, inhibits skeletal muscle myosin II in an ensemble motility assay and myoVa in a processivity assay. In contrast, Tpm3.1-P5, in which P5 is encoded by exon 6a, only affects myoVa. In the ensemble motility assay myosin II is insensitive to the mutations in exon 6a expressed in Tpm3.1. Striated muscle tropomyosins express only exon 6b, whereas smooth muscle and non-muscle tropomyosins express either exon 6a or 6b [Geeves et al., 2015]. *In vitro* studies of Tpm1.1 have shown that exon 6 can affect actin affinity [Hammell and Hitchcock-DeGregori, 1997; Pittenger and Helfman, 1992], but our results herein demonstrate that exon 6 encodes additional regulatory function.

Mutations in period P1, encoded by exon 1a in Tpm1.1 and 1b in Tpm3.1, have no observed effect on the function of either myosin (Figure 2, Figure 4). The first part encoded by exon 1 binds the C terminus of tropomyosin and tropomodulin [Colpan et al., 2013], and includes an actin binding site [Barua et al., 2013], but the function of the second half, the site of the P1 mutations, is unknown.

In conclusion, the work reported here identifies common and isoform-specific regulatory motifs in tropomyosins that control myosin function and advances discussion of the role of tropomyosin in encoding actin filaments for specific cellular functions. We suggest that tropomyosins and myosins are designed to work together in an optimal fashion, where tropomyosin can designate actin tracks to recruit or exclude specific myosins. Once bound, tropomyosin may directly regulate myosin function and/or recruit additional actin filament proteins, for example, troponin T in striated muscles. We suggest that functional studies of myosin may be enhanced by the inclusion of an appropriate tropomyosin on the actin filament. We propose two myosin-tropomyosin pairings, striated myosin II with muscle-specific Tpm1.1 (both expressed in muscles regulated by troponin) and myosin Va with Tpm3.1. Cellular studies including high-resolution microscopy would help define these and other tropomyosin-myosin pairings in cells.

Materials and Methods

DNA construction and protein purification

Mutations were made in rat Tpm1.1 cDNA (P1 and P7 mutants) (encoded by the *TPM1* gene; exons 1a-2b-3-4-5-6b-7-8-9a) with an Ala-Ser extension at the N terminus, and in rat Tpm3.1 (P1–P7 mutants) (encoded by the *TPM3* gene; exons 1b-3-4-5-6a-7-8-9d) cloned in pET11d for expression in *E. coli*. Recombinant AS-Tpm1.1 binds well to actin in the absence of troponin, unlike recombinant unacetylated-Tpm1.1 [Monteiro et al., 1994]. Mutations were made using oligonucleotides and their reverse complements using two-stage PCR as previously described [Singh and Hitchcock-DeGregori, 2003]. The oligonucleotides were synthesized by Integrated DNA Technologies (IDT), Coralville, IA. The mutations were verified by sequencing the full coding sequence of the DNA at Genewiz, South Plainfield, NJ. The Tpm3.1-P3 and P4 mutants were made by Mutagenex, Inc., Piscataway, NJ. Mutants were expressed in *E. coli* BL21(DE3) cells and purified as previously described [Singh and Hitchcock-DeGregori, 2003]. The tropomyosins were reduced with 1 mM DTT by heating at 56 °C for 5 minutes prior to all experiments.

Actin was purified from acetone powder of chicken pectoral skeletal muscle [Hitchcock-DeGregori et al., 1982]. Tropomyosin and actin concentrations were determined by measuring the difference spectrum of tyrosine [Singh and Hitchcock-DeGregori, 2003]. Skeletal myosin was prepared from chicken pectoral muscle [Winkelman et al., 1993]. Monoclonal antibody 10F12.3 that reacts specifically with chicken skeletal muscle myosin subfragment 2 was prepared and characterized as described previously [Winkelman and Lowey, 1986; Winkelman et al., 1983].

Truncated mouse myosin Va-HMM with a C-terminal biotin tag for attachment to streptavidin Qdots, followed by a FLAG-tag to facilitate affinity purification, were expressed and purified as previously described [Armstrong et al., 2012].

Ensemble motility assays

The ensemble motility assays were done as previously described [Barua et al., 2012; Winkelman et al., 1995]. Chicken skeletal myosin was flash frozen and stored in liquid nitrogen in 150 µL aliquots and thawed in ice followed by dead head removal. To remove dead heads, the skeletal myosin was combined with 1 mg/ml F-actin and 5 mM MgATP in high salt buffer (HSB) (25 mM imidazole, pH 7.6, 0.3 M KCl, 4 mM MgCl₂, 5 mM DTT) and centrifuged at 90,000 rpm for 20 minutes at 4°C in a Beckman TLA 100.2 rotor. This procedure pellets the F-actin and 5–10% of the total myosin, including inactive myosin that is not dissociated from actin by ATP (dead heads). The clear myosin supernatant was recovered and used within 0–16 hours of dead head removal.

Nitrocellulose-coated glass cover slips were incubated with 0.15 mg/ml of a monoclonal antibody that reacts specifically with chicken pectoral muscle myosin subfragment 2 (10F12.3), followed by incubation with 1% BSA for blocking. Skeletal myosin was diluted in HSB supplemented with 1% BSA (HSB/BSA) to a final concentration of 40 µg/ml. The antibody-coated coverslips were incubated with 40 µg/ml myosin for ~2 hours in a

humidified chamber at 4 °C, followed by washes with HSB/BSA, and then with motility buffer (25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl₂, 1 mM MgATP, 5 mM DTT).

The myosin-coated coverslips were transferred to a 15 µl drop of 2 nM rhodamine-phalloidin-labeled actin (chicken skeletal muscle) or actin-Tpm (tropomyosin concentration was 2 µM for all motility assays, except where indicated) in a modified motility buffer (25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl₂, 7.6 mM MgATP, 50 mM DTT, 0.5% methyl cellulose, 0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, 2.3 mg/ml glucose) in a small parafilm ring fixed on an alumina slide with vacuum grease. This chamber was observed with an Olympus BH-2 microscope at a temperature of 27 °C. The movement of actin filaments from 2–5 minutes of continuous video was recorded from several fields for each assay condition and analyzed with semi-automated filament tracking programs as described in [Barua et al., 2012; Winkelmann et al., 1995]. The weighted probability of the filament velocity for hundreds of tracks was fitted to a Gaussian distribution to determine a mean velocity ± standard deviation (s.d.) for each experiment (Figure S2).

Single molecule processivity assay

Myosin Va-HMM was diluted to 0.4 µM in Buffer A (10 mM imidazole, pH 7.4, 300 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 10 mM DTT), mixed with a 2-fold molar excess of actin and 1 mM MgATP, and centrifuged for 15 min at 400,000 *xg* to remove any myosin unable to dissociate from actin in the presence of ATP. Tropomyosin was diluted 2-fold in Buffer B100 (10 mM imidazole, pH 7.4, 100 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 10 mM DTT) and clarified for 15 min at 15,000 *xg*. Processivity assays were performed at room temperature (~24°C) in 20 µl flow chambers. Flow cells were first incubated with 0.1 mg/ml *N*-ethylmaleimide-modified skeletal muscle myosin in Buffer A for 4 min, blocked with 1 mg/ml BSA in buffer B25 (10 mM imidazole, pH 7.4, 25 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 10 mM DTT) for 2 min, rinsed with three volumes buffer B25, incubated with 0.5 µM rhodamine-phalloidin-labeled actin filaments and 2 µM Tpm in Buffer B25 for 3 min. Flow cells were blocked again with 1 mg/ml BSA in buffer B25 supplemented with 2 µM Tpm for 2 min and then equilibrated with three volumes buffer C-150 (10 mM imidazole, pH 7.4, 150 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 50 mM DTT, 1 mg/ml BSA, 0.1 mg/ml CaM all [Krementsov et al., 2004], 1 mM MgATP, an oxygen scavenger system (3 mg/ml glucose, 0.1 mg/ml glucose oxidase, 0.18 mg/ml catalase), an ATP regenerating system (0.5 mM phosphoenolpyruvate and 100 units/ml pyruvate kinase)) and 2 µM Tpm. Concentration of Tpm3.1-P7 was 4 µM in this assay. To ensure single motor conditions, 655 nm streptavidin-coated quantum dots (Qdots, Invitrogen) were added to HMM in five-fold excess [Hodges et al., 2012]. Final dilution of motors gave a concentration of ~5 nM in buffer C-150 supplemented with 2 µM Tpm (4 µM for Tpm3.1-P7). The microscope setup has been described previously [Armstrong et al., 2012]. Images were taken at 10 Hz frame rate using 2×2 pixel binning. Image stacks were analyzed using ImageJ v1.44k (National Institutes of Health, Bethesda, MD)[Rasband, 1997–2016]. We required motors to move continuously for four consecutive frames to be analyzed. Qdot position vs. time was tracked using the MtrackJ plugin for ImageJ [Meijering et al., 2012]. Speeds were calculated as the distance traveled/time. Run length was calculated as total distance traveled between first and last tracked frame. Run lengths were not analyzed from any tracks starting outside the time

and space coordinates of the movie and for tracks terminating at the end of a filament or at the edge of the field of view. Statistical analysis was performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). The characteristic run lengths and speeds were estimated by least squares fitting to cumulative distribution functions. Run frequency was calculated as number of processive runs/movie/actin length/myosin concentration. Data were obtained from at least 2 independent experiments using two different preparations of myosin. A minimum of 3 fields per experiment, $22\ \mu\text{m} \times 22\ \mu\text{m}$, that contained $>90\ \mu\text{m}$ of actin and lasted 20s each was analyzed. Exact numbers of total fields and individual motors analyzed are listed in Supplemental Table 1. To normalize, the run frequency of myoVa on actin decorated with wt-Tpm was set to 1.

Kinetic measurements

Stopped-flow fluorescence experiments were carried out as described in [Heeley et al., 2006], using KinTek SF-2001 double mixing stopped-flow fluorimeter. Kinetic measurements of mdADP dissociation from actomyosinII and actomyosinVa were done by mixing myosin-mdADP with F-actin or F-actin-Tpm3.1 containing a 1 mM MgATP chase to prevent mdADP from rebinding to the myosin. mdADP dissociation was observed using an excitation at 360 nm and emission $>420\ \text{nm}$ using a sharp cutoff filter. Double mixing experiments were done by mixing $3\ \mu\text{M}$ myosinII-S1 with $2\ \mu\text{M}$ mdATP, waiting 2 seconds to form a steady state mixture of M-mdATP and M-mdADP-Pi, which was then mixed with 10 to $100\ \mu\text{M}$ F-actin or F-actin-Tpm3.1 solutions containing a 1 mM ATP chase. Experiments using $5\ \mu\text{M}$ fluorescent phosphate binding protein, and a phosphate mop to reduce free phosphate concentration, to measure the kinetics of phosphate dissociation from actomyosinVa-ADP-Pi directly was done using double mixing stopped flow as previously described [White et al., 1997]. Data traces of 500 points were averaged, and the observed rate constants were obtained by fitting the data to one or two exponential terms using the software package included with the KinTek stopped-flow. The dependence of k_{obs} upon [F-actin] or [F-actin-Tpm3.1] was fit to a hyperbolic equation using the Scientist statistical package (Micromath, Salt Lake City, Utah):

$$k_{\text{obs}} = k_{\text{max}} / (1 + (K_{\text{app}} / [\text{F-actin-Tpm} - 3.1]))$$

Actin-Tpm3.1 complexes were performed at molar ratios of 1:2.5 (Tpm:actin) and analyzed by SDS-PAGE to verify that Tpm3.1 was bound to actin (Figure S3).

Actin binding assays

Tropomyosin ($0.1\text{--}8\ \mu\text{M}$) was combined with $5\ \mu\text{M}$ F-actin and cosedimented at $20\ ^\circ\text{C}$ in 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl_2 , and 0.5 mM DTT at 60,000 rpm in a Beckman model TL-100 ultracentrifuge in a TLA-100 rotor [Hammell and Hitchcock-DeGregori, 1996]. The pellets and supernatants were analyzed by SDS-PAGE, stained with Coomassie blue, and scanned and analyzed using ImageScanner III densitometer with Labscan 6.0 and ImageQuant TL 7.0 image analysis softwares (GE Healthcare, Uppsala, Sweden). The free Tpm in the supernatants was calculated from standard curves for wildtype Tpm. The binding constant K_{app} and the Hill coefficient (αH) were determined by fitting the

experimental data to the Hill equation using Kaleidagraph, v4.1.2 (Synergy Software, Reading, PA):

$$\nu = (n[\text{Tpm}]^{\alpha H} K_{\text{app}}^{\alpha H}) / (1 + [\text{Tpm}]^{\alpha H} K_{\text{app}}^{\alpha H})$$

where ν = fraction maximal Tpm binding to actin (Tpm:actin), n = maximal Tpm bound, and $[\text{Tpm}] = [\text{Tpm}]_{\text{free}}$. The Tpm:actin ratio was normalized to 1 by dividing the Tpm:actin ratio observed at each Tpm concentration by the Tpm:actin ratio observed at saturation.

Circular dichroism (CD) measurements

Thermal stability measurements were made by following the ellipticity of 0.1 mg/ml Tpm at 222 nm as a function of temperature in 0.5 M NaCl, 10 mM sodium phosphate, pH 7.5, 1 mM EDTA and 1 mM DTT in an Aviv model 400 spectropolarimeter at the Robert Wood Johnson Medical School Circular Dichroism facility, Piscataway, NJ. The observed melting temperature (T_M) is defined as the temperature at which the ellipticity at 222 nm, normalized to a scale of 0 to 1, is equal to 0.5 [Greenfield and Hitchcock-DeGregori, 1995].

Modeling of actin-tropomyosin-myosin structure

The model was constructed by docking a 7 Å-resolution Tpm1.1 crystal structure (Protein Data Bank (PDB) ID 1C1G) [Whitby and Phillips, 2000] to a proposed binding site in a 8 Å-resolution rigor actin-Tpm-myosin S1 complex determined by cryo-EM (PDB ID 4A7L) [Behrmann et al., 2012]. The myosin 1E (myoE) S1 structure in the EM model was replaced by the 2 Å-resolution myosin Va-S1 (PDB ID 1OE9) [Coureux et al., 2003] and the 2.25 Å-resolution cardiac myosin II-S1 (PDB ID 4PA0) [Winkelmann et al., 2015] structures through alignment of the upper 50 K domains of with myoE. We used the cardiac myosin II-S1 structure because it is a more recent and better-resolved structure compared to the skeletal myosin II S1 structure. The root mean square deviation (r.m.s.d.) between the two structures is 0.70 Å for 558 Ca atoms [Winkelmann et al., 2015] and the interacting residues shown in the model are similar in both structures. A modification was made in the axial position of Tpm in the EM model by a structural alignment of the crystal structure of Tpm1.1 (PDB ID 1C1G) with the Tpm model in the actin-Tpm-myosin S1 complex, to optimize electrostatic interactions of the mutated residues in P3 and P6 with charged residues on myosin Va and myosin II, which resulted in an axial movement of the Tpm but maintaining the azimuthal alignment of Tpm on actin [Barua et al., 2012]. All alignments were carried out in PyMOL (the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) using align (for myosin alignments) and CEalign (for Tpm alignment) functions, which perform a sequence alignment followed by a structural superposition, and then carry out zero or more cycles of refinement in order to reject structural outliers found during the fit. Residue numbers 29–164 (for P3 mutations) and 179–284 (for P6 mutations) of the crystal structure of Tpm (1C1G) were aligned with the Tpm (residues 98–233) in the EM model (4A7L).

We did not use the more recent von der Ecken et al., 2016 [von der Ecken et al., 2016] cryo-EM structure of actin-Tpm-myosin complex because we had completed our modeling before

the publication of that paper. Since the position and resolution of the tropomyosin is the same as in the Behrman et al., 2012 structure, we did not see any benefit in re-constructing our models using the newer structure. In addition, the actomyosin interactions in the higher resolution von der Ecken et al., 2016 structure are not sufficiently different from the Behrman et al., 2012 structure to significantly affect our models, in which we replaced the myosin in the original cryo-EM structure with myosin Va and cardiac myosin II crystal structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.

Regulation of skeletal myosin II and myosin Va-HMM by Tpm1.1 and Tpm3.1 mutants.

The bars are grey for bare actin, blue for Tpm mutations that regulate both myosins, orange for mutations that have myosin-specific effects and black for WT Tpm and mutations with little to no effect. (A, B) Filament speeds for actin-Tpm1.1 or Tpm3.1 with skeletal myosin II from ensemble motility assays. The data for Tpm1.1-P3–P6 are from [Barua et al., 2012]. Values are mean $\pm$ s.d., $n = 3–16$ (Table 1). Assay conditions: 25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl₂, 7.6 mM MgATP, 50 mM DTT, 0.5% methyl cellulose, and an oxygen scavenger system; 27°C. (C, D) Run frequencies of myosin Va-HMM moving on actin-Tpm1.1 or Tpm3.1. Run frequencies, defined as the number of moving motors per actin length per μ M motor per time, are normalized to WT Tpm. Values are mean $\pm$ s.d., $n = 6–17$ (Table 2). Assay conditions: 10 mM imidazole, pH 7.4, 150 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 50 mM DTT, 1 mg/ml BSA, 0.1 mg/ml CaM all, 1 mM MgATP, an oxygen scavenger system, and an ATP regenerating system; 23°C. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ compared to WT Tpm (ANOVA with Fisher's LSD test)

Figure 3.

Rate of dissociation of mdADP from actin-myosin-S1-mdADP-Pi or actin-myosin-S1-mdADP in the presence and absence of Tpm3.1. (A) k_{fast} is the rate of dissociation of Pi (A-MII-mdADP-Pi → A-MII-mdADP + Pi → A-MII + mdADP) measured by the rate of the decrease in mdADP fluorescence after dissociation from myosin II-S1. (C) The rate of Pi dissociation (k_{obs}) from A-MVa-ADP-Pi was measured directly using fluorescent phosphate binding protein. (B, D) Dependence of k_{obs} , the rate of dissociation of mdADP (A-M-mdADP → A-M + mdADP) on actin or actin-Tpm3.1 concentration. The dependence of the rates upon actin or actin-Tpm3.1 concentration were fit to the equation $k_{obs} = k_{max} / (1 + K_{act}/[actin])$ to obtain values for the rate constants of Pi or mdADP dissociation at saturating actin concentration (k_{max}) and the binding affinities for actin or actin-Tpm3.1 (K_{act}). These values were used to calculate the second-order rate constants (k_{max}/K_{act}) of M-ADP-Pi or M-ADP binding to actin prior to product dissociation. In panel D, we have not reported K_{act} values since no data was collected at low concentrations of actin/ actin-Tpm3.1.

Experimental conditions: 5 mM MOPS, pH 7, 2 mM MgCl₂, 1 mM DTT, 20 mM KAc; 20°C.

	Skeletal Myosin II	Myosin Va		Skeletal Myosin II	Myosin Va
Tpm1.1-P1 (1a)	-4%	-7%	Tpm3.1-P1 (1b)	+7%	0%
Tpm1.1-P3	-60%	-20%	Tpm3.1-P3	-60%	-65%
Tpm1.1-P4	-40%	-25%	Tpm3.1-P4	-20%	-30%
Tpm1.1-P5 (6b)	-50%	-70%	Tpm3.1-P5 (6a)	-7%	-40%
Tpm1.1-P6	-80%	-80%	Tpm3.1-P6	-50%	-70%
Tpm1.1-P7 (9a)	+11%	-8%	Tpm3.1-P7 (9d)	+7%	-65%

Figure 4.

Summary of the effects of Tpm1.1 and Tpm3.1 mutants on the regulation of skeletal myosin II (filament speeds in ensemble motility assays) and myosin Va (run frequency in single molecule processivity assays). The values are percent change relative to WT Tpm. The boxes are shaded blue for Tpm mutations that regulate both myosins, orange for the mutations that have myosin-specific effects and white for little to no effect.

Figure 5.

Molecular model for actin-tropomyosin-myosin in the open state. The model was constructed by docking a 7 Å-resolution Tpm1.1 crystal structure (Protein Data bank (PDB) ID 1C1G)[Whitby and Phillips, 2000] in a 8 Å-resolution rigor actin-Tpm-myosin S1 complex determined by cryo-EM (PDB ID 4A7L)[Behrmann et al., 2012]. The *Dictyostelium* myosin 1E (myoE) S1 structure in the EM model was replaced by myosin Va-S1 (PDB ID 1OE9)[Coureux et al., 2003] (A, B) and striated myosin II-S1 (PDB ID 4PA0) [Winkelmann et al., 2015] (C, D) structures through alignment with the upper 50K domain of myoE. A modification was made in the axial position of Tpm by a structural alignment of the Tpm1.1 crystal structure with the Tpm model in the actin-Tpm-myosin S1 complex, to optimize electrostatic interactions of the mutated residues in P3 and P6 with charged residues on myosin Va and myosin II, which resulted in an axial movement of the Tpm while maintaining the azimuthal alignment of Tpm on actin (see Materials and Methods). Tropomyosin is in cyan, actin in green and the myosin structures show the N-terminal 25K domain (blue), the converter domain and lever arm helix (yellow), the lower 50K domain (red) and the upper 50K domain (orange). Mutated residues in P3: (A) E114, K118 and D121 of Tpm can interact with K353, E369 and R343 of myosin Va, respectively, and (C) E114, D121 and E122 of Tpm can interact with K397, K367 and K365 of myosin II, respectively. Mutated residues in P6: (B) K233 and E240 of Tpm can interact with E369 and R343 of myosin Va, respectively, and (D) E234 and E240 of Tpm can interact with K397 and K365 of myosin II, respectively.

Table 1

Ensemble motility assay data for skeletal muscle myosin II with tropomyosin mutants

	Speed ($\mu\text{m/s}$) ^a	Fraction moving (total number) ^b
Actin	4.7 $\pm$ 0.2 (n=16) ***	0.95 (250)
Tpm1.1-WT	2.7 $\pm$ 1.0 (n=12)	0.87 (210)
Tpm1.1-P1	2.6 $\pm$ 0.5 (n =4)	0.93 (260)
Tpm1.1-P3	1.0 $\pm$ 0.6 (n = 4) ^c ***	0.40 (155) ^c
Tpm1.1-P4	1.7 $\pm$ 0.7 (n = 3) ^c *	0.68 (260) ^c
Tpm1.1-P5	1.3 $\pm$ 0.1 (n = 3) ^c **	0.54 (140) ^c
Tpm1.1-P6	0.6 $\pm$ 0.5 (n = 4) ^c ***	0.29 (190) ^c
Tpm1.1-P7	3.0 $\pm$ 0.8 (n =4)	0.94 (280)
Actin	4.7 $\pm$ 0.2 (n=16) ***	0.95 (250)
Tpm3.1-WT	7.4 $\pm$ 0.9 (n=8)	0.95 (320)
Tpm3.1-P1	7.9 $\pm$ 0.5 (n=5)	0.96 (420)
Tpm3.1-P3	2.9 $\pm$ 0.6 (n=4) ***	0.78 (165)
Tpm3.1-P4	6.2 $\pm$ 0.2 (n=3) **	0.95 (275)
Tpm3.1-P5	6.9 $\pm$ 0.5 (n=4)	0.98 (280)
Tpm3.1-P6	3.5 $\pm$ 1.0 (n=6) ***	0.93 (240)
Tpm3.1-P7	7.9 $\pm$ 0.3 (n=3)	0.97 (260)

^aFilament speeds $\pm$ standard deviation (s.d.) of actin and actin-Tpm from ensemble motility assays. The n values are in parentheses.

*
p <0.1,

**
p <0.01,

p <0.001 compared to WT Tpm (ANOVA with Fisher's LSD test).

^bThe average values for the fraction of moving filaments. The average values for the total number of filaments are in parentheses.

^cFrom Barua *et al.*, 2012 [Barua *et al.*, 2012]. The speeds and fraction of moving filaments were 4.7 $\pm$ 0.2 $\mu\text{m/s}$ (0.95) and 3.1 $\pm$ 1.0 $\mu\text{m/s}$ (0.90) for actin and Tpm1.1-WT, respectively in Barua *et al.*, 2012.

Table 2

Single molecule processivity assay data for myosin Va-HMM with tropomyosin mutants

	Speed ($\mu\text{m/s}$) ^a	Run length (μm) ^b	Run frequency (normalized) ^c
Actin	0.87 ± 0.24 (n=106)	0.51 (0.47, 0.56) (n=102)	1.13 ± 0.23 (n=10)
Tpm1.1-WT	0.77 ± 0.31 (n=78)	0.43 (0.37, 0.51) (n=74)	1.00 ± 0.11 (n=9)
Tpm1.1-P1	0.72 ± 0.22 (n=76)	0.39 (0.35, 0.45) (n=68)	0.93 ± 0.31 (n=6)
Tpm1.1-P3	0.80 ± 0.39 (n=52)	0.60 (0.46, 0.85) (n=49)	0.80 ± 0.22 (n=9) *
Tpm1.1-P4	0.83 ± 0.30 (n=53)	0.37 (0.32, 0.44) (n=49)	0.74 ± 0.14 (n=6) *
Tpm1.1-P5	0.79 ± 0.29 (n=23)	0.63 (0.45, 1.10) (n=22)	0.32 ± 0.12 (n=6) ***
Tpm1.1-P6	0.85 ± 0.43 (n=16)	0.57 (0.34, 1.72) (n=15)	0.22 ± 0.11 (n=6) ***
Tpm1.1-P7	0.74 ± 0.26 (n=68)	0.29 (0.27, 0.31) (n=64)	0.92 ± 0.18 (n=6)
Actin	0.73 ± 0.28 (n=106)	0.50 (0.47, 0.53) (n=97)	0.61 ± 0.24 (n=17) ***
Tpm3.1-WT	0.68 ± 0.28 (n=105)	0.49 (0.46, 0.53) (n=90)	1.00 ± 0.25 (n=13)
Tpm3.1-P1	0.66 ± 0.19 (n=95)	0.54 (0.49, 0.59) (n=86)	1.00 ± 0.42 (n=17)
Tpm3.1-P3	0.60 ± 0.25 (n=96)	0.49 (0.45, 0.53) (n=77)	0.35 ± 0.13 (n=13) ***
Tpm3.1-P4	0.63 ± 0.22 (n=105)	0.45 (0.41, 0.50) (n=91)	0.72 ± 0.21 (n=13) **
Tpm3.1-P5	0.65 ± 0.24 (n=100)	0.56 (0.51, 0.61) (n=87)	0.58 ± 0.19 (n=15) ***
Tpm3.1-P6	0.59 ± 0.25 (n=106)	0.49 (0.45, 0.53) (n=84)	0.32 ± 0.17 (n=13) ***
Tpm3.1-P7	0.70 ± 0.25 (n=101)	0.45 (0.42, 0.48) (n=86)	0.34 ± 0.13 (n=14) ***

^aSingle molecule speeds $\pm$ s.d. of myosin Va-HMM on actin or actin-Tpm^bRun lengths of single molecule myosin Va-HMM moving on actin or actin-Tpm. Values in parentheses are 95% confidence intervals (ANOVA).^cRun frequencies of single myosin Va-HMM moving along actin or actin-Tpm shown as fold increase over WT Tpm $\pm$ s.d.*
p < 0.1,**
p < 0.01,***
p < 0.001 compared to WT Tpm (ANOVA with Fisher's LSD test)