A periodic pattern of evolutionarily-conserved basic and acidic residues constitutes the binding interface of actin-tropomyosin*

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*Running Title: Actin-tropomyosin interface

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Background: The interface of actin with tropomyosin, universal regulator of the actin filament is unknown.

Results: Mutagenesis of actin and tropomyosin revealed a pattern of residues required for complex formation in the closed state.

Conclusion: The results support models of the actin-tropomyosin filament in the absence of myosin and troponin.

Significance: A validated actin-tropomyosin model is required to understand regulation and disease mechanisms.

SUMMARY:
Actin filament cytoskeletal and muscle functions are regulated by actin binding proteins using a variety of mechanisms. A universal actin filament regulator is the protein, tropomyosin that binds end-to-end along the length of the filament. The actin-tropomyosin filament structure is unknown but there are atomic models in different regulatory states based on electron microscopy reconstructions, computational modeling of actin-tropomyosin, and docking of atomic resolution structures of tropomyosin to actin filament models. Here, we have tested models of the actin-tropomyosin interface in the “closed state” where tropomyosin binds to actin in the absence of myosin or troponin. Using mutagenesis coupled with functional analyses we determined residues of actin and tropomyosin required for complex formation. The sites of mutations in tropomyosin were based on an evolutionary analysis and revealed a pattern of basic and acidic residues in the first-halves of the periodic repeats (periods) in tropomyosin. In periods P1, P4 and P6, basic residues are most important for actin affinity, in contrast to periods P2, P3, P5 and P7, where both basic and acidic residues or predominantly acidic residues contribute to actin affinity. Hydrophobic interactions were found to be relatively less important for actin binding. We mutated actin residues in subdomains 1 and 3 (D25-E334-K326-K328) that are poised to make electrostatic interactions with the residues in the repeating motif on tropomyosin in the models. Tropomyosin failed to bind mutant actin filaments. Our mutagenesis studies provide the first experimental support for the atomic models of the actin-tropomyosin interface.

Tropomyosin (Tm) is a regulator of the actin cytoskeleton that is involved in diverse cellular functions including muscle contraction, cytokinesis, intracellular transport, and cell migration (1-3). Tropomyosin is a two-chained α-helical coiled coil protein that associates end-to-end to form continuous strands on both sides of the actin filament. Binding of Tm to actin regulates the stability and functions of actin filaments in most eukaryotic muscle and non-muscle cells ranging from mammals to fungi. In striated muscle, Tm and troponin (Tn) regulate muscle contraction by Ca2+-dependent regulation of the binding of myosin heads (myosin S1) to actin filaments. Mutations in Tm cause skeletal and cardiomyopathies (4,5). In non-muscle cells,
Tm regulates actin filament dynamics as well as interaction of actin with actin binding proteins, including, myosin, tropomodulin, formin, Arp2/3 and ADF-cofilin (1,3). Elimination of both tropomyosin genes in budding yeast is lethal. Although the ability to bind actin filaments is a universal function of Tm, the specific residues of actin and Tm that constitute the binding interface of the actin-Tm complex remain unknown.

Striated muscle αTm spans the length of seven actin monomers in the actin filament and was proposed to have seven quasi-equivalent periodic repeats (periods, P1-P7) with a pattern of residues within each period postulated to be actin binding sites (6,7). Subsequent biochemical and biophysical studies defined features of the Tm molecule that are important for actin binding and regulation including the molecular ends, an uninterrupted heptad repeat, regions of local instability at the coiled coil interface characterized by the presence of small non-polar residues such as Ala, and a sequence pattern on the surface of the coiled coil that was proposed as a recognition site for actin binding (8-11). Previous studies have also shown that individual periodic repeats of Tm contribute in different ways to actin binding and regulatory function (11-15). Atomic models based on electron microscopy (EM) reconstructions, three-dimensional fluorescence resonance energy transfer (FRET) analysis and computational modeling of actin-Tm have revealed the positions of Tm on the actin filament in the absence and presence of Tn±Ca2+, and myosin S1 (16-19). However, there is no high-resolution, residue-specific structural information available for the actin-Tm binding interface.

We previously reported an evolutionary analysis of Tms from 26 species ranging from cnidarians to vertebrates and determined the evolutionarily conserved residues of Tm (20). Evolutionarily conserved residues at b, c, or f surface positions of the coiled coil heptad repeat of Tm were mutated to Ala within the first-half or the second-half of periods P2-P6. All Tm mutations, except one, were at charged residues indicating that binding is primarily electrostatic in nature. Mutations in the first-half of P2 (P2-1-KDDE), P4 (P4-1-RKV) and P5 (P5-1-REEE) showed the largest reduction in actin affinity (>4-fold), inferring that these mutations include residues in actin binding sites. A structural model constructed to assess the structural relevance of these mutations showed potential periodic interactions of mutated sites in Tm with residues in subdomains 1 and 3 of successive actin monomers of the filament and is consistent with other models (18,21). Mutations in the second halves of the periods primarily affect actomyosin regulation (22).

In the present work, we refined our analysis of conserved surface residues (20) in order to determine features of individual periods that form actin binding sites on Tm. In addition, we extended the analysis to include periods P1 and P7. Our goal was to determine the actin-tropomyosin interface residues in the absence of proteins such as troponin and myosin that modify the intrinsic actin affinity of tropomyosin. We determined that actin binding sites on Tm follow an alternating pattern of basic and basic-acidic residues in each periodic repeat. In P1, P4 and P6, basic residues are most important for actin affinity, in contrast to P2, P3, P5 and P7, where both basic and acidic residues or mostly acidic residues contribute to actin affinity. Hydrophobic interactions are relatively less important for actin binding. To test the models of the actin-Tm interface (18,20,21), we mutated actin residues in subdomains 1 and 3 (D25-E334-K326-K328) that are poised to make electrostatic interactions with the residues in the repeating motif on Tm. Tropomyosin failed to bind mutant actin filaments, in support of the models. The velocity of the mutant filament was reduced in an actomyosin motility assay, suggesting that these residues are also involved in actin-myosin interaction.

EXPERIMENTAL PROCEDURES
DNA construction and protein purification-
Mutations were made in rat striated α-tropomyosin cDNA with an Ala-Ser extension at the N-terminus, cloned in pET11d for expression in E. coli. (23). Recombinant AS-αTm binds well to actin in the absence of troponin, unlike recombinant unacetylated-αTm. Mutations were made using oligonucleotides and their reverse complements using two-stage PCR as previously described (9). The mutations were verified by sequencing of the DNA at the DNA Core Facility at RWJMS and Genewiz. Mutants were expressed in E. coli. BL21(DE3) cells and purified as previously described (9). Actin was purified from
acetone powder of chicken pectoral skeletal muscle actin (24). Tm and actin concentrations were determined by measuring the difference spectrum of tyrosine (9). The molecular weights of the purified proteins were verified by electrospray mass spectrometry at the Keck Biotechnology Resource Lab, Yale University.

**Smooth muscle actins**- Recombinant baculovirus were prepared according to established protocols (25). Human vascular smooth muscle actins (ACTA2) were tagged at the C-terminus with a 6xHIS, using the method described in Noguchi et al. (26). Infected SF9 cells were harvested after 72 h and lysed in 1 M Tris-HCl, pH 7.5 at 4°C, 0.6 M KCl, 0.5 mM MgCl2, 4% Triton X-100, 1 mg/ml Tween 20, 0.5 mM Na2ATP, 1 mM DTT, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 5 mM benzamidine, and 5 µg/ml leupeptin for 2.5 h (40 ml/1 billion cells). After clarification at 178,000 x g for 45 min, the supernatant was dialyzed overnight into 10 mM HEPES, pH 7.5, 0.3 M NaCl, 0.2 mM CaCl2, 0.25 mM Na2ATP, 7 mM β-mercaptoethanol, and 1 µg/ml leupeptin. The dialysate was clarified at 25,000 x g for 30 minutes and then incubated with HIS resin. The column was washed first with the dialysis buffer without imidazole and then with the dialysis buffer containing 10 mM imidazole. Protein was eluted with dialysis buffer containing 50 mM imidazole. Pure fractions were combined, concentrated using an Amicon concentrator, and dialyzed into G buffer (5 mM Tris, pH 8.26 at 4°C, 0.2 mM CaCl2, 0.1 mM NaN3, 0.5 mM DTT, 0.2 mM Na2ATP, 1 µg/ml leupeptin). The tag was cleaved off of the actin and the tag separated from actin on a MonoQ column, essentially as described in Noguchi et al. (26). Pure actin was dialyzed against G-buffer for 2 days, clarified, and polymerized by the addition of 2 mM MgCl2 and 0.1 mM KCl.

**Actin binding assays**- Tropomyosin (0.1-8 µM) was combined with 5 µM chicken skeletal F-actin or 3 μM human vascular smooth F-actin and cosedimented at 20°C in 200 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM MgCl2, and 0.5 mM DTT at 60,000 rpm in a Beckman model TL-100 ultracentrifuge in a TLA-100 rotor (27). 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. The free Tm in the supernatants was calculated from standard curves for WT-Tm. The binding constant Kapp and the Hill coefficient (αH) were determined by fitting the experimental data to the Hill equation (Equation 1) (9) using Kaleidagraph:

\[
\nu = (n[Tm]_{\alpha H}K_{app}^{\alpha H}) / (1 + [Tm]_{\alpha H}K_{app}^{\alpha H}) 
\]

where ν= fraction maximal Tm binding to actin, n= maximal Tm bound, and [Tm] = [Tm]free. The Tm:actin ratio was normalized to 1 by dividing the Tm:actin ratio obtained from densitometry by the Tm:actin ratio observed at saturation.

**Circular dichroism (CD) measurements**- Thermal stability measurements were made by following the ellipticity of 1.5 µM Tm 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 RWJMS CD facility. The observed melting temperature (TM) 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 (28).

**EM imaging**- Actin was diluted to 15 µg/ml in 10 mM Tris, pH 7.5, 2 mM MgCl2, 0.5 mM DTT, 100 mM NaCl and 0.1 mM ATP and applied to freshly prepared carbon-coated butvar grids and negatively stained with 1% uranyl acetate in water. Images were recorded with a Phillips CM12 transmission electron microscope operating at 80 kV and 45,000x magnification with an AMT XR611 digital system.

**In vitro motility assays**- The in vitro motility assays were done as described in (22,29). Nitrocellulose-coated glass cover slips were incubated with 0.15 mg/ml of a monoclonal antibody that reacts specifically with myosin subfragment 2 (anti-S2 mAb; 10F12.3), followed by incubation with 1% BSA for blocking. 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. The coverslips were washed with HSB/BSA, followed by washes with motility

3
buffer (25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl₂, 1 mM MgATP, 5 mM DTT), then transferred to a 15 µl drop of 2 nM rhodamine-phalloidin-labeled smooth muscle WT or mutant actin in a modified motility buffer (25 mM imidazole, pH 7.6, 25 mM KCl, 4 mM MgCl₂, 1 mM MgATP, 5 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. The movement of actin filaments from 1-2 minutes of continuous video was recorded from several fields for each assay condition and analyzed with semi-automated filament tracking programs as described previously (22,30,31).

RESULTS
Tropomyosin mutant design- In a previous study, evolutionarily conserved residues of Tm at surface b, c, or f heptad repeat positions in periods P2-P6 were mutated to alanine with 3-4 residues mutated in each construct in a 284-residue rat striated αTm with a N-terminal Ala-Ser extension to mimic acetylation (20). The Ala-Ser Tm functions normally in in vitro analyses as well as in reconstituted fibers (23,32,33). Here, we have carried out further mutagenesis to determine the contribution of individual residues within the first half of periods 1-7 to actin binding in order to refine our understanding of the actin binding sites on the surface of Tm. The additional mutations are all of well-conserved sites, and include single-site mutations and tests of the importance of the pattern of conserved surface residues. A pattern of basic and acidic surface residues is conserved in the first-half of P1-P7 at positions f, b and f of the heptad repeat (shown by the blue and red boxes in Figure 1). At the first f position (blue box, Figure 1), there is a basic residue (K6, K48, R90, R125, R167 and R244) in each period except P6 (A209). However in P6, there are two basic residues at adjacent b and c positions (K205 and K213) that may be analogous to the basic residues at position f. At the b and f positions C-terminal to the basic residues (red boxes, Figure 1), there are acidic residues in all periods except position b in P4 (Q135).

Here we have introduced the following mutations: the basic and acidic residues at the f, b, f positions in P1 and P7 were mutated to Ala, the R90A and D100A mutations were added to the P3-1-EE mutant, and the K205A, D219A and E223A mutations were added to the K213A (P6-1-K) mutant (20) (Figure 1). We carried out single residue mutations to Ala at the basic residues in position f: K6, K48, R125, R167 and R244, and at K205-K213 in P6 (P6-1-KK), to separate the contributions of basic and acidic residues to actin affinity. We also mutated hydrophobic residues in P4 (V129) and P5 (V170) to serine to test the contribution of hydrophobic interactions to actin binding.

Actin affinity of tropomyosins- The actin affinity (K_{app}) of the Tm mutants was determined by cosedimentation with skeletal muscle F-actin (Figure 2, Table 1). All mutations reduced actin affinity >2-fold. The reduction in actin affinity caused by a mutation, either individual or in combination with others, was taken to indicate the contribution of that residue to an actin binding site on Tm. Mutations of non-conserved residues in these regions, or conserved residues in the second halves of the periods have little to no effect on actin affinity (20). The P4-1-RKVE and P4-1-RKV mutants have similar actin affinities, indicating that the E139A mutation does not affect actin binding.

The loss in affinity shown by basic residue mutants in P1 (K6A), P4 (R125A) and P6 (P6-1-KK) was similar to that of the basic-acidic residue mutants (P1-1-KED, P4-1-RKV and P6-1-KKDE) indicating the actin binding sites in P1, P4 and P6 are primarily the basic residues. In contrast, basic residue mutations in P2 (K48A), P3 (P3-1-REE), P5 (R167A) and P7 (R244A) accounted for about half or less than half of the loss in affinity shown by the basic-acidic residue mutants (P2-1-KDDE, P3-1-REDE, P5-1-REEE and P7-1-RDD), indicating the contribution of both basic and acidic residues to actin binding in these periods. A mutant with four basic residues mutated (48-90-125-167A) had ~7-fold decrease in affinity similar to R125A confirming that R125 has the largest contribution to actin affinity amongst the four mutated basic residues. Mutation of hydrophobic residues in P4 (V129S) and P5 (V170S) had a ~2-3-fold effect on actin affinity indicating the relatively smaller contribution of hydrophobic interactions to actin binding. These results indicate...
that the binding interface of actin-Tm is primarily electrostatic in nature and the actin binding sites on Tm alternate between basic residues and basic-acidic residues in each period (Figure 6).

**Thermal stability of tropomyosins** - The thermal unfolding of the Tm α-helical coiled coil was determined using circular dichroism (CD) by measuring the ellipticity at 222 nm from 0-65 °C. The thermal unfolding data did not show any major change in the stability of the Tm mutants compared to WT (Table 1, Figure 3). The overall melting temperature (T_M, temperature at which tropomyosin is 50% unfolded) of the mutants was within 2-3 °C of WT T_M. Previous studies have shown that stabilization of interface regions of Tm that contain Ala and other destabilizing residues leads to decreased actin affinity. There is no correlation between the reduced actin affinities of our mutants and the small changes in thermal stability, allaying concern that the Ala mutations may affect actin affinity because of changes in the global stability of the Tms. The small effect of the mutations on T_M indicates that there is no global stabilization, implying that the reduced actin affinity of mutants reflects binding specificity and is not a consequence of helical stabilization.

**Smooth muscle actins** - EM and computational models of the actin-tropomyosin complex suggest that the binding sites for Tm on actin lie in subdomains 1 and 3. Based on the results with Tm mutants in this and our previous study and models of actin-Tm (18,20,21), we hypothesize that the pattern of basic and acidic residues in the first halves of Tm’s periodic repeats interact with oppositely charged residues, D25, E334, K326 and K328, of actin (Figure 4). In order to test this, we made an actin mutant, D25-E334-K326-K328A, with four residues mutated to Ala. The mutations were made in a human vascular smooth muscle α-actin (ACTA2) construct and expressed in Drosophila Sf9 cells. Electron microscopy (EM) of negatively stained samples of actin showed normal actin filament morphology for both WT and D25-E334-K326-K328A smooth muscle actins (Figure 5A).

The binding of WT and D25-E334-K326-K328A actin to WT Tm was measured by cosedimentation (Figure 5B). WT actin bound to WT Tm with a binding constant (K_{app}) of 7 x10^6 M^{-1}. In contrast, the D25-E334-K326-K328A mutant did not show measurable binding to WT Tm, implying that one or more of the mutated actin residues in contain a binding site for Tm. The filament speeds of WT and D25-E334-K326-K328A actin were determined using in vitro motility assays on skeletal myosin surfaces at a myosin density that allows maximal velocity of skeletal actin filaments (Figure 5C). The velocity of D25-E334-K326-K328A was ~25% slower relative to WT actin and the fraction of moving filaments decreased from ~80% for WT to ~35% for D25-E334-K326-K328A actin. These results indicate that the actin mutations may impair myosin binding and/or ATPase activation.

**DISCUSSION**

The present work relates functional analyses to computational models and reports significant progress towards our goal to establish the actin-Tm interface in the absence of an atomic resolution structure. Our goal in the present study was to determine the actin-tropomyosin interface residues in the absence of proteins such as troponin and myosin that modify the intrinsic actin affinity of tropomyosin. We have defined common features of the interface while gaining insight into period-specific features of actin binding sites on Tm. The current work builds on our previous analysis of evolutionarily-conserved sites. Here we included periods P1 and P7, and mutated additional conserved sites in the first half of P2-P6 that are well-conserved but that fell above the arbitrary cutoff of ω ≤ 0.015 in our previous study (20).

An earlier model of the periodic repeats identified a pattern of seven conserved surface residues proposed to be actin binding sites (7) that has been tested and supported (10,11). Of these seven residues, a basic residue in an f position at the beginning of the repeat, followed by acidic residues in b and f positions in an i-to-i + 4 relationship, are the most consistent features of the repeat (Figure 1), and are shown to be important in our iterative analyses. Here we show that the basic residue participates in all seven sites with an alternating pattern of sites with basic (P1, P4, P6) and basic-acidic residues (P2, P3, P5, P7) (Figure 6). The results underscore previous reports of particular contributions of each period to actin
affinity and thin filament function (11-15). The relatively minor involvement of hydrophobic residues is consistent with the primarily electrostatic nature of actin-Tm binding. Without an atomic resolution structure, however, our present proposal does not preclude the involvement of additional residues in the actin-Tm interface.

We note that the basic and basic-acidic motifs of the proposed actin binding sites include non-canonical features of the Tm coiled coil interface that are themselves highly-conserved and may therefore be crucial for local conformation and interaction with actin. P1 and P5 have “alanine clusters” that cause local flexibility (21,34), a feature important for actin affinity (9-11). Two highly-conserved non-canonical interface residues (D137 in a d position and E218 in an a position) that cause bends in the molecule (21,35,36), are in P4 and P6 respectively.

Our finding that Tm fails to bind to actin mutated at D25-E334-K326-K328 supports the available molecular models for actin-Tm in the closed state (18,20,21). Therefore, one or more of the four mutated actin residues are binding sites for Tm. The slight (~25%) inhibition of velocity of the actin mutant relative to WT actin in the motility assay also indicates the involvement of the mutated residues in myosin binding or ATPase activation. Atomic models of actin-myosin show actin residues K326 and E334 as myosin binding residues (16,37). The hypothesis that Tm and myosin compete for a common site on actin in the steric blocking model (38-41) is supported by the reduced velocity of filament speed in an actin-myosin motility assay.

In summary, the binding sites of Tm and actin based on our mutagenesis studies support atomic models of actin-Tm (18,20,21), where in the absence of Tn and myosin, Tm is predicted to bind to the actin filament at subdomains 1 and 3 and inhibit the binding of myosin to actin. Three sites in the pattern of basic-acidic residues in Tm, E62 in P2, R167 in P5, and R244 in P7, are the sites of disease-causing mutations in humans (4,5). Some of the actin mutations studied here are also known to be disease-causing mutations in the human skeletal muscle actin gene (ACTA1) (42), are lethal in yeast, and cause abnormal muscle structure in Caenorhabditis and Drosophila (43-46). It is likely that severely impaired binding of Tm to actin filaments may explain these phenotypes.
REFERENCES:
1. Gunning, P., O'Neill, G., and Hardeman, E. (2008) Tropomyosin-based regulation of the actin cytoskeleton in time and space. *Physiol Rev* **88**, 1-35
2. Pollard, T. D., and Cooper, J. A. (2009) Actin, a central player in cell shape and movement. *Science* **326**, 1208-1212
3. Wang, C. L., and Coluccio, L. M. (2010) New insights into the regulation of the actin cytoskeleton by tropomyosin. *Int Rev Cell Mol Biol* **281**, 91-128
4. Kee, A. J., and Hardeman, E. C. (2008) Tropomyosins in skeletal muscle diseases. *Adv Exp Med Biol* **644**, 143-157
5. Wieczorek, D. F., Jagatheesan, G., and Rajan, S. (2008) The role of tropomyosin in heart disease. *Adv Exp Med Biol* **644**, 132-142
6. McLachlan, A. D., and Stewart, M. (1976) The 14-fold periodicity in alpha-tropomyosin and the interaction with actin. *J Mol Biol* **103**, 271-298
7. Phillips, G. N., Jr. (1986) Construction of an atomic model for tropomyosin and implications for interactions with actin. *J Mol Biol* **192**, 128-131
8. Hitchcock-DeGregori, S. E. (2008) Tropomyosin: function follows structure. *Adv Exp Med Biol* **644**, 60-72
9. Singh, A., and Hitchcock-DeGregori, S. E. (2003) Local destabilization of the tropomyosin coiled coil gives the molecular flexibility required for actin binding. *Biochemistry* **42**, 14114-14121
10. Singh, A., and Hitchcock-DeGregori, S. E. (2006) Dual requirement for flexibility and specificity for binding of the coiled-coil tropomyosin to its target, actin. *Structure* **14**, 43-50
11. Singh, A., and Hitchcock-DeGregori, S. E. (2007) Tropomyosin's periods are quasi-equivalent for actin binding but have specific regulatory functions. *Biochemistry* **46**, 14917-14927
12. Landis, C., Back, N., Homsher, E., and Tobacman, L. S. (1999) Effects of tropomyosin internal deletions on thin filament function. *J Biol Chem* **274**, 31279-31285
13. Hitchcock-DeGregori, S. E., Song, Y., and Greenfield, N. J. (2002) Functions of tropomyosin's periodic repeats. *Biochemistry* **41**, 15036-15044
14. Kawai, M., Lu, X., Hitchcock-Degregori, S. E., Stanton, K. J., and Wandling, M. W. (2009) Tropomyosin period 3 is essential for enhancement of isometric tension in thin filament-reconstituted bovine myocardium. *J Biophys* **2009**, 380967
15. Singh, A., and Hitchcock-DeGregori, S. E. (2009) A peek into tropomyosin binding and unfolding on the actin filament. *PLoS One* **4**, e6336
16. Behrmann, E., Muller, M., Penczek, P. A., Mannherz, H. G., Manstein, D. J., and Raunser, S. (2012) Structure of the rigor actin-tropomyosin-Myosin complex. *Cell* **150**, 327-338
17. Lehman, W., and Craig, R. (2008) Tropomyosin and the steric mechanism of muscle regulation. *Adv Exp Med Biol* **644**, 95-109
18. Li, X. E., Tobacman, L. S., Mun, J. Y., Craig, R., Fischer, S., and Lehman, W. (2011) Tropomyosin position on F-actin revealed by EM reconstruction and computational chemistry. *Biophys J* **100**, 1005-1013
19. Miki, M., Makimura, S., Saitoh, T., Bunya, M., Sugahara, Y., Ueno, Y., Kimura-Sakiyama, C., and Tobita, H. (2011) A three-dimensional FRET analysis to construct an atomic model of the actin-tropomyosin complex on a reconstituted thin filament. *J Mol Biol* **414**, 765-782
20. Barua, B., Pamula, M. C., and Hitchcock-DeGregori, S. E. (2011) Evolutionarily conserved surface residues constitute actin binding sites of tropomyosin. *Proc Natl Acad Sci U S A* **108**, 10150-10155
21. Brown, J. H., Zhou, Z., Reshetnikova, L., Robinson, H., Yammani, R. D., Tobacman, L. S., and Cohen, C. (2005) Structure of the mid-region of tropomyosin: bending and binding sites for actin. *Proc Natl Acad Sci U S A* **102**, 18878-18883
22. Barua, B., Winkelmann, D. A., White, H. D., and Hitchcock-DeGregori, S. E. (2012) Regulation of actin-myosin interaction by conserved periodic sites of tropomyosin. *Proc Natl Acad Sci U S A* **109**, 18425-18430
23. Monteiro, P. B., Lataro, R. C., Ferro, J. A., and Reinach, F. C. (1994) Functional alpha-tropomyosin produced in Escherichia coli. A dipeptide extension can substitute the amino-terminal acetyl group. J Biol Chem 269, 10461-10466
24. Hitchcock-De Gregori, S. E., Mandala, S., and Sachs, G. A. (1982) Changes in actin lysine reactivities during polymerization detected using a competitive labeling method. J Biol Chem 257, 12573-12580
25. O'Reilly, D. R., Miller, L.K. and Luckow, V.A. (1992) Baculovirus Expression Vectors, A Laboratory Manual, W.H. Freeman and Co., New York
26. Noguchi, T. Q., Gomibuchi, Y., Murakami, K., Ueno, H., Hirose, K., Wakabayashi, T., and Uyeda, T. Q. (2010) Dominant negative mutant actins identified in flightless Drosophila can be classified into three classes. J Biol Chem 285, 4337-4347
27. Hammell, R. L., and 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
28. Greenfield, N. J., and Hitchcock-DeGregori, S. E. (1995) The stability of tropomyosin, a two-stranded coiled-coil protein, is primarily a function of the hydrophobicity of residues at the helix-helix interface. Biochemistry 34, 16797-16805
29. Winkelmann, D. A., Bourdieu, L., Ott, A., Kinose, F., and Libchaber, A. (1995) Flexibility of myosin attachment to surfaces influences F-actin motion. Biophys J 68, 2444-2453
30. Bourdieu, L., Magnasco, M. O., Winkelmann, D. A., and Libchaber, A. (1995) Actin filaments on myosin beds: The velocity distribution. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Topics 52, 6573-6579
31. Wang, Q., Moncman, C. L., and Winkelmann, D. A. (2003) Mutations in the motor domain modulate myosin activity and myofibril organization. J Cell Sci 116, 4227-4238
32. Lu, X., Tobacman, L. S., and Kawai, M. (2003) Effects of tropomyosin internal deletion Delta23Tm on isometric tension and the cross-bridge kinetics in bovine myocardium. J Physiol 553, 457-471
33. Lu, X., Tobacman, L. S., and Kawai, M. (2006) Temperature-dependence of isometric tension and cross-bridge kinetics of cardiac muscle fibers reconstituted with a tropomyosin internal deletion mutant. Biophys J 91, 4230-4240
34. Brown, J. H., Kim, K. H., Jun, G., Greenfield, N. J., Dominguez, R., Volkmann, N., Hitchcock-DeGregori, S. E., and Cohen, C. (2001) Deciphering the design of the tropomyosin molecule. Proc Natl Acad Sci U S A 98, 8496-8501
35. Nitanai, Y., Minakata, S., Maeda, K., Oda, N., and Maeda, Y. (2007) Crystal structures of tropomyosin: flexible coiled-coil. Adv Exp Med Biol 592, 137-151
36. Sumida, J. P., Wu, E., and Lehrer, S. S. (2008) Conserved Asp-137 imparts flexibility to tropomyosin and affects function. J Biol Chem 283, 6728-6734
37. Lorenz, M., and Holmes, K. C. (2010) The actin-myosin interface. Proc Natl Acad Sci U S A 107, 12529-12534
38. Haselgrove, J. C. (1972) X-ray evidence for a conformational change in the actin-containing filaments of vertebrate striated muscle. Cold Spring Harbor Symp. Quant. Biol. 37, 341-352
39. Huxley, H. E. (1972) Structural changes in actin and myosin-containing filaments during contraction. Cold Spring Harbor Symp. Quant. Biol. 37, 361-376
40. Parry, D. A., and Squire, J. M. (1973) Structural role of tropomyosin in muscle regulation: analysis of the x-ray diffraction patterns from relaxed and contracting muscles. J Mol Biol 75, 33-55
41. Vibert, P., Craig, R., and Lehman, W. (1997) Steric-model for activation of muscle thin filaments. J Mol Biol 266, 8-14
42. Laing, N. G., Dye, D. E., Wallgren-Pettersson, C., Richard, G., Monnier, N., Lillis, S., Winder, T. L., Lochmuller, H., Graziano, C., Mitani-Rosenbaum, S., Twomey, D., Sparrow, J. C., Beggs, A.
H., and Nowak, K. J. (2009) Mutations and polymorphisms of the skeletal muscle alpha-actin gene (ACTA1). *Hum Mutat* **30**, 1267-1277

43. Drummond, D. R., Hennessey, E. S., and Sparrow, J. C. (1991) Characterisation of missense mutations in the Act88F gene of Drosophila melanogaster. *Mol Gen Genet* **226**, 70-80

44. Frieden, C., Du, J., Schriefer, L., and Buzan, J. (2000) Purification and polymerization properties of two lethal yeast actin mutants. *Biochem Biophys Res Commun* **271**, 464-468

45. Waterston, R. H., Hirsh, D., and Lane, T. R. (1984) Dominant mutations affecting muscle structure in Caenorhabditis elegans that map near the actin gene cluster. *J Mol Biol* **180**, 473-496

46. Wertman, K. F., Drubin, D. G., and Botstein, D. (1992) Systematic mutational analysis of the yeast ACT1 gene. *Genetics* **132**, 337-350

47. Fujii, T., Iwane, A. H., Yanagida, T., and Namba, K. (2010) Direct visualization of secondary structures of F-actin by electron cryomicroscopy. *Nature* **467**, 724-728

48. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **25**, 1605-1612

49. Whitby, F. G., and Phillips, G. N., Jr. (2000) Crystal structure of tropomyosin at 7 Angstroms resolution. *Proteins* **38**, 49-59
Actin-tropomyosin interface

Acknowledgements- We thank Brinda Desai and Yazan Alkhawam for technical assistance.

FOOTNOTES
This work was supported by NIH grant GM093065 to S.E.-H.-D and HL110869 to KMT.

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The abbreviations used are: Tm, tropomyosin; Tn, troponin; myosin S1, myosin subfragment 1; EM, electron microscopy; FRET, fluorescence resonance energy transfer; CD, circular dichroism; T_{M}, melting temperature

FIGURE LEGENDS
FIGURE 1. Tropomyosin mutations at conserved surface residues. The rat striated αTm sequence showing conserved b, c, and f residues that were mutated to Ala in the first-half of periods P1-P7. The names of the mutants on the left indicate the basic (blue), acidic (red) or hydrophobic (grey) residues that were mutated in the first-half (1) of each period (P1-P7). The pattern of basic and acidic residues at positions f, b, and f are indicated by the blue (position f, basic residues) and red (positions b and f, acidic residues) boxes.

FIGURE 2. Actin affinity of tropomyosin mutants measured by cosedimentation with F-actin. Tropomyosin (0.1-8 µM) was combined with 5 µM chicken skeletal α-actin and sedimented at 20ºC in 200 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, and 0.5 mM DTT. Stoichiometric binding of one Tm per seven actins is represented by fraction maximal binding of 1. The data for each mutant and WT was obtained from 2-6 independent experiments. The K_{app} values are reported in Table 1.

FIGURE 3. Thermal stability of Tm mutants measured by circular dichroism. Fraction folded as measured by relative ellipticity at 222 nm as a function of temperature. The Tm concentration was 1.5 µM in 0.5 M NaCl, 10 mM sodium phosphate (pH 7.5), 1 mM EDTA, and 1 mM DTT. The fraction folded is relative to the mean residue ellipticity at 0 °C, where the proteins were fully folded. The T_{M} values are reported in Table 1.

FIGURE 4. Model for actin-tropomyosin interaction. The model was constructed by docking a 2.3 Å Tm crystal structure (blue, PDB ID 2B9C) (21) including periods P4 and P5, and a 6.6 Å F-actin structure (green, PDB ID 3MFP) (47) as described in Barua et al. (20). The model shows potential electrostatic and hydrophobic interactions between Tm residues in P4 and P5 and actin residues. The zoomed portion shows the actin-Tm interface in P5. The model was constructed using the UCSF Chimera package (48).

FIGURE 5. Smooth muscle WT and D25-E334-K326-K328A (mutant) α-actins. A. EM images of F-actin negatively stained with uranyl acetate. B. Affinity of actins for WT Tm measured by cosedimentation assay. Tropomyosin (0.1-8 µM) was combined with 3 µM F-actin and sedimented at 20ºC in 200 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM MgCl₂, and 0.5 mM DTT. The K_{app} values are reported in Table 2. C. Filament speed of actins in in vitro motility assays. The values are mean ± SD from two experiments. An anti-myosin subfragment 2 monoclonal antibody was bound to nitrocellulose-coated glass coverslips and then incubated with 40 µg/ml chicken skeletal myosin at 4ºC for 2 h. The coverslips were transferred to 15 µL drops of 2 mM rhodamine-phalloidin labeled smooth muscle F-actins in 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), and an oxygen scavenger system (0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 2.5 mg/ml glucose) and 1-2 minutes of continuous video of movement of actin filaments were recorded from several fields for each experiment at 27 ºC.
FIGURE 6. Summary of contributions of basic, acidic and hydrophobic residues in individual periodic repeats to tropomyosin function. A. Tropomyosin residues at conserved surface positions that were mutated to Ala in the first-half of periods P1-P7 (basic residues in blue, acidic residues in red and hydrophobic residues in grey). The numbers indicate the reduction in actin affinity of the mutants compared to WT Tm ($K_{WT}/K_{mut}$). In P1, P4 and P6, primarily basic residues contribute to actin affinity (blue boxes), in contrast to P2, P3, P5 and P7, where both basic and acidic or mostly acidic residues contribute to actin affinity (red boxes). The hydrophobic residues have a smaller contribution to actin affinity. B. Tropomyosin mutations shown in the 7 Å striated muscle αTm structure (1C1G) (49).
## TABLE 1. Actin affinity and T<sub>M</sub> from the ellipticity at 222 nm (CD) of tropomyosin mutants

| Mutant       | <i>K<sub>app</i></sub><sup>a</sup> (x10<sup>-6</sup> M<sup>-1</sup>) | <i>K<sub>WT</sub></i>/<i>K<sub>mut</sub></i> | CD<sup>b</sup> | Mutant       | <i>K<sub>app</i></sub><sup>a</sup> (x10<sup>-6</sup> M<sup>-1</sup>) | <i>K<sub>WT</sub></i>/<i>K<sub>mut</sub></i> | CD<sup>b</sup> |
|--------------|---------------------------------|---------------------|-----------|--------------|---------------------------------|---------------------|-----------|
| WT<sup>c</sup> | 2.1 ±0.1                         | -                   | 49        | R167A       | 0.9 ±0.1                         | 2                   | 47        |
| K6A          | 0.6 ±0.0                         | 4                   | 49        | P5-1-REEE<sup>c</sup> | 0.4 ±0.0                         | 5                   | 51        |
| K48A         | 1.1 ±0.0                         | 2                   | 49        | K213A (P6-1-K) | 1.3 ±0.1                         | 2                   | 49<sup>p</sup> |
| P2-1-KDDE<sup>c</sup> | 0.5 ±0.0                         | 4                   | 49        | P6-1-KK     | 0.6 ±0.0                         | 4                   | 49        |
| P3-1-EE<sup>c</sup> | 1.2 ±0.1                         | 2                   | 49        | P6-1-KKDE   | 0.6 ±0.0                         | 4                   | 51        |
| P3-1-REE     | 0.9 ±0.0                         | 2                   | 49        | P7-1-RDD    | 0.2 ±0.0                         | 10                  | 52        |
| P3-1-REDE    | 0.1 ±0.0                         | >10                 | 48        | R244A       | 0.4 ±0.0                         | 5                   | 49        |
| R125A        | 0.2 ±0.1                         | 10                  | 48        | 48-90-125-167A | 0.3 ±0.0                         | 7                   | 47        |
| P4-1-RKV<sup>c</sup> | 0.3 ±0.0                         | 7                   | 47        | V129S       | 0.9 ±0.0                         | 2                   | 49        |
| P4-1-RKVE<sup>c</sup> | 0.4 ±0.0                         | 5                   | 49        | V170S       | 0.7 ±0.0                         | 3                   | 49        |

<sup>a</sup>The values for <i>K<sub>app</sub></i> shown with standard error. The data were fit to the Hill equation, and the <i>K<sub>app</sub></i> are those reported by Kaleidagraph.

<sup>b</sup>T<sub>M</sub> is the temperature at which the ellipticity at 222 nm, normalized to a scale of 0 to 1, is equal to 0.5.

<sup>c</sup>The <i>K<sub>app</sub></i> and T<sub>M</sub> values are from Barua <i>et al</i>. (20)

## TABLE 2. Tropomyosin binding affinity and filament speeds of actin

| Smooth muscle actin | <i>K<sub>app</sub></i><sup>a</sup> (x10<sup>-6</sup> M<sup>-1</sup>) | Speed<sup>b</sup> (µm/s) | % moving /total number<sup>c</sup> |
|---------------------|---------------------------------|---------------------|----------------------------------|
| WT                  | 7.0 ±0.1                         | 3.0 ±0.1            | 81 /163                          |
| D25-E334-K326-K328A | ND                              | 2.2 ±0.1            | 34 /178                          |

<sup>a</sup>The values for <i>K<sub>app</sub></i> shown with SE. The data were fit to the Hill equation, and the <i>K<sub>app</sub></i> are those reported by Kaleidagraph.

ND means <i>K<sub>app</sub></i> was not measurable, less than 0.01 x10<sup>6</sup> M<sup>-1</sup>.

<sup>b</sup>Filament speeds shown with SD of actin filaments from <i>in vitro</i> motility assays (n=2).

<sup>c</sup>The average values for the percent moving filaments and the total number of filaments (n=2).
Figure 2.
Figure 4.
Figure 5.
Figure 6.

|     | Basic          | Basic + Acidic | Hydrophobic |
|-----|----------------|----------------|-------------|
| P1  | 4x K6A         | 4x (K6A + E16-D20A) |             |
| P2  | 2x K48A        | 4x (K48A + D55-D58-E62A) |             |
| P3  | 2x (R90A + E97-E104A) | 10x (R90A + E97-D100-E104A) |             |
| P4  | 9x R125A       | 7x (R125A + K128-V129S-E139A) | 2x V129S   |
| P5  | 2x R167A       | 5x (R167A + E177-E181-E184A) | 3x V170S   |
| P6  | 4x (K213-K205A) | 4x (K213-K205A + D219-E223A) |             |
| P7  | 5x R244A       | 10x (R244A + D254-D258A) |             |
A periodic pattern of evolutionarily-conserved basic and acidic residues constitutes the binding interface of actin-tropomyosin
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*J. Biol. Chem.* published online February 18, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.451161

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