Lysine acetylation of F-actin decreases tropomyosin-based inhibition of actomyosin activity

William Schmidt#, Aditi Madan, D. Brian Foster, and Anthony Cammarato*

From the Department of Medicine at Johns Hopkins University School of Medicine, Baltimore, MD 21205

#Present address: William Schmidt, Rivier University, Science and Innovation Center 231, 420 Main St., Nashua, NH 03063; wschmidt@rivier.edu

*Corresponding author: Anthony Cammarato: Department of Medicine, Johns Hopkins University, Ross Bldg. 1050, Baltimore, MD 21205; acammar3@jhmi.edu; Tel. 410-955-1807 Fax. 410-502-2558

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ABSTRACT

Recent proteomics studies of vertebrate striated muscle have identified lysine acetylation at several sites on actin. Acetylation is a reversible post-translational modification (PTM) that neutralizes lysine’s positive charge. Actin’s positively charged residues, particularly K326 and K328, are predicted to form several, critical electrostatic interactions with tropomyosin (Tpm) that promote its binding and bias Tpm to an azimuthal location where it impedes myosin attachment. The troponin (Tn) complex also influences Tpm’s position along filamentous (F-) actin as a function of Ca\(^{2+}\) to regulate exposure of myosin-binding sites and, thus, myosin cross-bridge recruitment and force production. Interestingly, K326 and K328 on sarcomeric actin are among the documented acetylated residues. Using an acetic anhydride-based labeling approach, we showed that excessive, non-specific actin acetylation did not disrupt characteristic F-actin-Tpm binding. However, it significantly reduced Tpm-mediated inhibition of myosin attachment as reflected by increased F-actin-Tpm motility that persisted in the presence of Tn and submaximal Ca\(^{2+}\). Furthermore, decreasing the extent of chemical acetylation, to presumptively target highly-reactive K326 and K328, also resulted in less inhibited F-actin-Tpm, implying that modifying these residues only, influences Tpm’s location and, potentially, thin filament regulation. To unequivocally determine the residue-specific consequences of acetylation on Tn-Tpm-based regulation of actomyosin activity, we assessed the effects of K326Q and K328Q Ac-mimetic actin on Ca\(^{2+}\)-dependent, in vitro motility parameters of reconstituted thin filaments (RTFs). Incorporation of K328Q actin significantly enhanced Ca\(^{2+}\) sensitivity of activation relative to control. Together our findings suggest that actin acetylation, particularly K328, modulates muscle contraction via disrupting inhibitory Tpm positioning.

INTRODUCTION

Muscle contraction is driven by cyclical, force-generating interactions between myosin-containing thick and actin-based thin filaments (1, 2). Conversely, relaxation occurs when actomyosin binding is inhibited and/or suppressed by actin-associated regulatory proteins, Tn and Tpm, on thin filaments, or by the sequestration of myosin heads on thick filaments (1-4). Tn is a trimer composed of inhibitory (TnI), Tpm-binding (TnT), and Ca\(^{2+}\) -binding (TnC) subunits (1, 2, 5). Each Tn-Tpm holoregulatory complex associates with seven actin protomers of the thin filament to confer Ca\(^{2+}\) sensitivity of contraction (1, 2). Under low Ca\(^{2+}\), TnI restricts Tpm to the blocked “B-state” position along F-actin where it sterically inhibits myosin binding (1, 2, 5). This effect is attenuated as free intracellular Ca\(^{2+}\) rises and binds to TnC. Ca\(^{2+}\)-TnC binding relieves TnI-mediated constraints on Tpm...
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and facilitates its azimuthal movement to the closed “C-state”, partial exposure of myosin-binding sites on actin, and weak myosin binding. The transition of myosin heads from a weak to strong F-actin-bound state induces additional Tpm movement to the open “M-state”, triggering cooperative thin filament activation and force generation. Hence, contraction demands both Tn- and myosin-dependent shifts in Tpm position.

Tpm is an alpha-helical, coiled-coil dimer consisting of seven tandem “quasi-equivalent”, pseudo-repeating motifs that bind adjacent actin monomers (6-8). It self-polymerizes head-to-tail to form a continuous cable that extends the entire length of F-actin (9-13). Tpm dimers individually bind with weak affinity, orchestrated by favorable electrostatic contacts between residues of each actin protomer and Tpm pseudorepeat, yet the polymerized cable binds with exceedingly high affinity (10, 14-19). This contrasting behavior, described as “gestalt-binding”, bestows on Tpm the requisite ability to easily shift its azimuthal position over the F-actin surface to regulate myosin binding to the thin filament without risk of catastrophic detachment (10, 20). Mutations that disrupt the weak, local binding of Tpm to F-actin, as suggested by distorted, computationally-derived F-actin-Tpm electrostatic energy landscapes, may minimally impact F-actin-Tpm global binding affinity, yet elicit significant changes in Tpm’s ability to properly impede actomyosin binding and cross-bridge cycling (21-23). Therefore, perturbed F-actin-Tpm electrostatic interactions could affect Tpm’s preferred binding position, and/or its regulatory switching between the B-, C-, and M-states along F-actin and, thus, Tpm-mediated regulation of actomyosin activity.

EM reconstructions and in silico-generated atomic models of Tpm bound to F-actin have identified Tpm’s potential default Apo- or “A-state” binding location, where the regulatory strand is positioned such that it sterically hinders myosin binding in the absence of Tn (12, 15, 20, 24). K326 and K328 of actin are predicted to form highly-favorable electrostatic contacts with Tpm and stabilize its native inhibitory configuration (12, 15, 17-19, 25, 26). These associations dominate the computed F-actin-Tpm interaction energy landscape and, together with TnI-actin interactions, are essential for establishing the B-state (5, 12, 15, 22, 27). Modification or mutation of K326 or K328 on actin, or residues in their vicinity, have been shown to alter contractile regulation and induce cardiac and skeletal myopathies (21, 22, 28-30).

Recently, ten distinct lysines of actin were identified as targets of acetylation in independent proteomics assessments of healthy, vertebrate cardiac and skeletal muscle (31, 32). Of those ten residues, five, including K326 and K328, were reported to be acetylated in both studies. Lysine acetylation is a reversible PTM where the transfer of an Ac group to the residue’s side-chain terminal amine, neutralizes its positive charge. Since K326 and K328 on actin are likely critical for establishing favorable electrostatic interactions with Tpm, “masking” the charges could destabilize F-actin-Tpm binding and/or inhibitory positioning, resulting in increased actomyosin cross-bridge formation and enhanced force production. In fact, mimicking acetylation, by replacing the charged lysines at positions 326 and 328 with uncharged glutamines in Drosophila indirect flight muscle (IFM) altered flight ability and stimulated IFM fiber destruction due to excessive, myosin-dependent force generation (30).

In vivo, actin acetylation levels may be distinctly modulated. For example, fluctuations in intracellular concentrations of Ac-CoA stimulate changes in lysine acetylation levels via mass action (33, 34). Alternatively, enzymatic-mediated changes in acetylation require targeting of actin by particular lysine acetylase(s) (KATs) and/or deacetylase(s) (KDACs). Despite evidence implicating specific enzymes that may perform this role in vivo (35-37), to the best of our knowledge no group has provided evidence of successful enzymatic acetylation of purified, sarcomeric actin in vitro. On the other hand, in vitro chemical acetylation of actin is eminently feasible (38-40). For example, Hitchcoek-DeGregori et al. discovered, via a competitive labeling method, that K326 and K328 on F-actin displayed a high
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propensity for acetylation and were preferentially modified relative to all other lysines (38).

Here, we hypothesize that actin K326 and/or K328 acetylation decreases Tpm-based inhibition of myosin binding by direct disruption of F-actin-Tpm electrostatic contacts and resultant Tpm mispositioning, which ultimately manifests as enhanced Ca$^{2+}$ sensitivity in regulated thin filaments. To test our hypothesis, we implemented a two-pronged approach that determined the effects of 1) indiscriminate and probabilistic, but preferential, actin lysine acetylation on global F-actin-Tpm binding, and on the inherent inhibitory positioning of Tpm along F-actin in the absence and presence of Tn and 2) residue-specific “pseudo”-acetylation of K326 or K328 on Ca$^{2+}$-mediated regulation of RTFs. Our results suggest that while K326 and K328 acetylation does not affect collective F-actin-Tpm affinity, K328 acetylation does lessen Tpm’s intrinsic ability to inhibit myosin cross-bridge binding, which contributes to enhanced thin filament Ca$^{2+}$ sensitivity.

RESULTS

According to the Behrmann et al. F-actin-Tpm-myosin rigor structure (41), K328 of actin forms a salt bridge with E286 of myosin (Fig. 1A). Acetylation of K328, and masking of its positive charge, would predictably disrupt this interaction, which could potentially affect myosin binding and/or cross-bridge cycling. We pan-acetylated F-actin with acetic anhydride to increase actin-normalized lysine acetylation levels ~290-fold (Fig.’s 1B, C, S1) and employed in vitro motility assays to assess its gross effect on actomyosin interactions (Fig. 1D). Sliding velocities of control and acetylated F-actin did not significantly differ at any of the myosin concentrations tested, suggesting that, in general, actomyosin cross-bridge cycling is seemingly unaffected by actin acetylation.

To rule out the possibility that the lack of an effect on F-actin sliding velocities was because K328 remained unmodified, we employed MS to confirm which lysines were acetylated following chemical treatment (Table 1). Specifically, MS detected 22 unique spectral matches to 19 unique trypic peptide sequences. These corresponded to 12 distinct acetylation sites, most of which are common to multiple actin gene products, including cardiac, skeletal, and cytoplasmic isoforms. Therefore, the absence of an acetylation-induced effect on actomyosin cycling and, specifically, F-actin propulsion speeds, could not be attributed to a failure of K328 to be chemically modified in vitro.

K326 and K328 of seven consecutive actin protomers are predicted to interact with negatively charged residues of every pseudo-repeat along Tpm’s entire length (12, 15, 20, 25, 26) (Fig. 2A). Previously, site-directed mutagenesis of D25, E334, K326, and K328 of human smooth muscle actin was shown to result in a complete loss of bacterially-expressed Tpm binding, which suggests that the lysines may contribute to global F-actin-Tpm binding (19). Therefore, we determined the effect of F-actin acetylation on the binding of tissue-purified cardiac Tpm via co-sedimentation (Fig. S2). We postulated that modifying the preponderance of actin lysines, especially K326 and K328, would potentially decrease maximum F-actin-Tpm binding ($B_{max}$) and/or affinity ($K_d$). However, $B_{max}$ and $K_d$ of Tpm for control ($B_{max} = 0.15 \pm 0.01, K_d = 0.21 \pm 0.03 \mu M$) and acetylated ($B_{max} = 0.14 \pm 0.01, K_d = 0.15 \pm 0.04 \mu M$) F-actin did not significantly differ (Fig. 2B). Thus, extensive chemical modification of actin lysines did not appear to alter either the number of available Tpm binding sites or the affinity of the polymerized Tpm cable for F-actin.

It is well established that F-actin-Tpm association is highly influenced by buffer ionic strength due to the considerable contribution of electrostatic interactions to binding (42-44). Therefore, actin acetylation may alter the buffer salt sensitivity of Tpm binding due to marked reduction in positively charged lysines, specifically K326 and/or K328. We performed F-actin-Tpm co-sedimentation assays at varying KCl concentrations to determine if actin acetylation modifies the effect of buffer salt on F-actin-Tpm binding (Fig. 2C). At 40 and 200 mM KCl, control (112 ± 6.3% and 85.4 ± 6.9%
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respectively) and acetylated (113 ± 2.2% and 70.4 ± 4.7% respectively) F-actin bound roughly equivalent amounts of Tpm, while increasing KCl concentration to 500 mM and above resulted in near-complete lack of Tpm binding to both F-actin types. This supports the idea that, despite extensive lysine charge neutralization of acetylated F-actin, including K326 and K328, collective Tpm binding to F-actin remains largely intact.

Next, we assessed whether Tpm was located along acetylated F-actin at or near its typical inhibitory A-state position and could, therefore, alter actomyosin cycling and/or the extent of myosin recruitment, which influence muscle contraction (45, 46). We first determined in vitro motility velocities of control and acetylated F-actin and F-actin-Tpm at varying myosin concentrations (Fig.’s 3A, B). To facilitate interpretation regarding the degree to which Tpm reduces filament gliding speed, we normalized control and acetylated F-actin-Tpm velocities at each myosin concentration to their respective F-actin velocities, subtracted normalized values from one, and multiplied by 100 to quantify the percent of Tpm-based inhibition (Fig. 3C). As observed previously (47-50), Tpm significantly decreased ($p < 0.0001$) both control (Fig. 3A) and acetylated (Fig. 3B) F-actin velocities, however, actin acetylation had no significant effect on Tpm-mediated reduction of velocity (Fig. 3C). To further assess the effect of Tpm on actomyosin binding, we quantified the percentage of moving control and acetylated actin and actin-Tpm filaments (Fig.’s 3D, E). Consistent with an inhibitory effect on velocity (Fig.’s 3A-C), Tpm significantly decreased ($p < 0.0001$) the percent of motile control (Fig. 3D) and acetylated (Fig. 3E) F-actin. Yet, in contrast, acetylated F-actin-Tpm movement was significantly elevated (i.e. less inhibited ($p < 0.0001$)) relative to control (Fig. 3F) while, as before (Fig. 1D), the PTM had no impact on F-actin movement (Fig.’s 3D, E). Therefore, Tpm’s intrinsic ability to inhibit myosin recruitment to acetylated F-actin, as opposed to cross-bridge turnover rate, was significantly attenuated compared to control F-actin (Fig.’s 3C, F). In addition, the greatest difference (i.e. a 63% relative increase) in acetylated vs. control F-actin-Tpm movement occurred at the lowest myosin concentration tested (12.5 µg/ml). Under these conditions, myosin-dependent, propagated movement of Tpm and exposure of proximal binding sites is normally, relatively minimal. Thus, Tpm likely remains predominantly in the A-state, along control F-actin and the extent of myosin binding is determined by Tpm’s innate ability to block myosin attachment, which appears compromised following modification of F-actin lysines. Taken together, the data suggest that actin acetylation increases cross-bridge accessibility by impairing Tpm’s capacity to adequately impede actomyosin binding when Tpm alone is bound to F-actin. This effect could potentially translate into less inhibited thin filaments in the presence of Tn and Ca$^{2+}$.

We subsequently assessed the ability of acetylated actin to serve as the backbone of a filament that responds to Ca$^{2+}$. We reconstituted thin filaments by adding Tn-Tpm to control and acetylated F-actin and determined velocity and percent moving filaments at three distinct Ca$^{2+}$ levels. Given the more pronounced relative effect of actin acetylation on F-actin-Tpm movement under low myosin concentrations, we elected to measure RTF activation at 25 µg/ml myosin. At low Ca$^{2+}$ (pCa 9), movement of control and acetylated actin-containing RTFs was limited and discontinuous and, therefore, unquantifiable under our motion criteria (see Supporting information) (Fig. 4A). At high Ca$^{2+}$ (pCa 4) the average velocity and percent filaments moving of acetylated actin-containing RTFs (1.11 ± 0.17 µm/s and 40.5 ± 3.4% respectively) were comparable to control (1.19 ± 0.21 µm/s and 40.1 ± 3.9% respectively) (Fig. 4A, B). However, when assessed at a submaximal, activating level of steady-state Ca$^{2+}$ (pCa 6.5), akin to an amount transiently present in actively contracting human myocytes (51-53), average acetylated vs. control actin-containing RTF velocities (0.27 ± 0.01 µm/s vs. 0.18 ± 0.02 µm/s respectively) along with percent filaments moving (24.75 ± 1.63% vs. 20.11 ± 1.45% respectively) were significantly elevated ($p < 0.05$) (Fig. 4C).
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These results are consistent with enhanced F-actin-Tpm movement (Fig.’s 3D-F) and indicate that acetylated actin is capable of forming a Ca\(^{2+}\)-responsive thin filament that is less inhibited and hypersensitive to Ca\(^{2+}\).

Since K50, K61, K315, K326, and K328 are commonly observed actin acetylation sites in vertebrates (31, 32), they may be the most physiologically relevant. As previously noted, K326 and K328 are critical for establishing Tpm’s inhibitory state in vitro and in vivo (12, 15, 20, 22, 30), therefore, it stands to reason that acetylation of these residues specifically would have the greatest contribution to Tpm mispositioning and increased actomyosin accessibility. Therefore, we attempted to more precisely resolve the effects of modifying K326 and K328 by altering the acetylation reaction to favorably target these residues (Fig. S3). Based on previous measures of relative reactivities of actin lysines (38), we proceeded under the premise that performing the acetylation reaction with sub-stoichiometric amounts of acetic anhydride would preferentially target K326 and K328 and, thus, any observed changes in Tpm-dependent inhibition of myosin binding could predominantly be attributed to modifying these residues. F-actin-Tpm in vitro motility was performed following treatment of actin with either sub- or supra-stoichiometric amounts of acetic anhydride (Fig. 5A). Sub-stoichiometric acetic anhydride treatment resulted in significantly greater (p < 0.0001) F-actin-Tpm movement relative to control. Furthermore, movement of F-actin-Tpm containing sub-stoichiometric-treated actin was statistically equivalent to F-actin-Tpm movement containing supra-stoichiometric-treated actin (Fig. 5A). Therefore, actin acetylation with sub-stoichiometric levels of acetic anhydride that favors K326 and K328 acetylation recapitulates the impact of actin treated with supra-stoichiometric levels of acetic anhydride, which is less discriminate. This might indicate that K326 and/or K328 acetylation preferentially contribute to the observed increases in the percent of F-actin-Tpm movement, and RTF movement and velocity, which is in agreement with corroborating structural and in silico modeling data of the F-actin-Tpm interface (12, 15, 17-19, 25, 26).

Therefore, actin K326 and/or K328 modification, specifically, potentially modulates regulation of filaments replete with Tn-Tpm complexes.

Drosophila serves as a vehicle for transgenic actin production that can be purified in ample amounts needed for in vitro studies (22, 49, 54). The Act88F IFM actin isoform shares 92% identity with human ACTA skeletal actin, and previous in vitro studies have confirmed that the two behave indistinguishably in vitro (22, 55). Similarly, the Act57B cardiac actin isoform is 93% identical to human ACTC. To unambiguously assess the site-specific impact of K326 or K328 modification on Ca\(^{2+}\)-dependent thin filament regulation, we created transgenic Drosophila fly lines that express WT, K326Q, or K328Q Ac-mimetic (charge-neutralizing, yet sterically similar) Act57B actin in the IFM (30). Transgenic actin comprised roughly 15% of total IFM actin (Fig S4), which was isolated and reconstituted into thin filaments using vertebrate cardiac Tn and Tpm. Ca\(^{2+}\) responsiveness of Drosophila actin-containing RTFs was determined via regulated in vitro motility at 100 µg/ml myosin as described previously (22). Maximum velocities of RTFs containing transgenic “pseudo”-acetylated actin did not deviate more than 3% from respective transgenic WT actin-containing RTFs (Fig.’s 5B, C). Furthermore, K326Q actin-containing RTFs exhibited no change in Ca\(^{2+}\) sensitivity ([Ca\(^{2+}\)]\(_{50}\) = 0.42 ± 0.027 µM and 1.7 ± 0.21) (Fig. 5B). However, K328Q actin-containing RTFs were significantly hypersensitive (p < 0.0001) to Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{50}\) = 0.56 ± 0.032 µM) relative to WT ([Ca\(^{2+}\)]\(_{50}\) = 0.87 ± 0.044 µM) with no change in cooperativity (h = 1.7 ± 0.22 respectively) (Fig. 5C). Increased Ca\(^{2+}\) sensitivity in K328Q actin-containing RTFs is noteworthy for three reasons: 1) it is consistent with our previous data indicating increased myosin binding to acetylated F-actin-Tpm and RTFs at submaximal Ca\(^{2+}\) (Fig.’s 3, 4); 2) it suggests that K328 acetylation alone is sufficient to cause Tpm mispositioning and, therefore, less Ca\(^{2+}\) is required for RTF activation; and 3) it reveals the potential
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for K328 acetylation specifically to modulate and enhance thin filament Ca\(^{2+}\) sensitivity in vivo.

DISCUSSION

This is the first study to assess the in vitro effects of actin acetylation on Tpm- and Tn-Tpm-mediated inhibition of actomyosin binding and cycling activity. Interestingly, in vitro chemical acetylation of F-actin appeared to have little effect on Tpm binding as determined by co-sedimentation assays. Since a previous study reported that the simultaneous substitution of actin D25, E334, K326, and K328, with alanines abolished F-actin-Tpm binding (19), we anticipated, at a minimum, a decrease in global Tpm binding affinity. However, no such reduction was observed under our experimental conditions. This discrepancy might be due to one or more of the following factors. First, negative effects on Tpm binding caused by alanine substitution-induced perturbations in the F-actin-Tpm topological landscape might exceed those elicited by either glutamine substitutions or Ac group transfer and charge neutralization. Second, relatively few positively charged K326 and/or K328 residues may be sufficient for collective Tpm binding. Third, disparities resulting from expression of recombinant rat striated α-Tpm and human smooth muscle actin compared to tissue-purified bovine cardiac Tpm and rabbit skeletal actin, as employed here, could differentially affect Tpm binding to the modified actins (16, 19, 20, 56, 57). Fourth, positively charged arginines on actin potentially minimize the loss in binding due to lysine charge neutralization. Last, negatively charged D25 and/or E334 are also important contributors to global F-actin-Tpm binding affinity. Discriminating among these and additional possible mechanisms will require further investigation.

According to the results obtained using our in vitro motility protocol, acetylation had no effect on rabbit skeletal F-actin sliding speeds across all concentrations of rabbit skeletal myosin tested. This corroborates the notion that the modification does not overtly impact actomyosin interactions and cross-bridge turnover rate in the absence of Tpm (Fig. 1D). In the presence of the regulatory strand, however, the number of motile actin-Tpm filaments significantly increased relative to control (Fig.’s 3D-F), and the increase was significant under conditions that favored pan-acetylation of actin lysines or, potentially, under that which favored modification of only K326 and K328 (38) (Fig. 5A). These findings added credence to the thought that K326 and/or K328 modification might mitigate the ability of Tpm to inherently block myosin binding to actin.

Hyperacetylation of actin did not adversely affect thin filament stability or trigger disassembly since increasing actin acetylation ~290-fold did not hamper in vitro reconstitution of or render RTFs unresponsive to Ca\(^{2+}\), as motility was completely absent at pCa 9 (Fig. 4A) and comparable to control RTFs at pCa 4 (Fig. 4A, B). Therefore, at steady-state, and in the presence of low or maximal Ca\(^{2+}\) (58-60), acetylation did not seemingly impact Tn’s intrinsic ability to bind F-actin-Tpm and establish the thin filament regulatory B- or M-states. However, at submaximal, activating pCa 6.5, both the percent motile and average velocity of acetylated actin-containing RTFs were significantly higher than control (Fig. 4C), consistent with reduced inhibition of myosin binding. Thus, despite a loss in positive surface charge, acetylated actin was capable of reconstituting into, and forming the molecular backbone of, a Ca\(^{2+}\)-responsive thin filament that evidently is hypersensitive to Ca\(^{2+}\) as indicated by increased myosin recruitment and propulsion speed at pCa 6.5.

Based on F-actin-Tpm results supporting the idea that K326 and/or K328 actin acetylation was largely responsible for attenuating Tpm-based inhibition (Fig. 5A), we reconstituted thin filaments containing Ac-mimetic K326Q or K328Q actin and tested the residue-specific effects of the modification on Ca\(^{2+}\) regulation of motility by measuring maximum sliding speeds, Ca\(^{2+}\) sensitivity, and cooperativity of activation (Fig.’s 5B, C). We found that RTFs containing ~15% K328Q “pseudo”-acetylated actin (30) were hypersensitive to Ca\(^{2+}\) (Fig. 5C). Enhanced Ca\(^{2+}\) sensitivity is consistent with increased acetylated F-actin-Tpm movement (Fig.’s 3, 5A) and enhanced activation of acetylated actin-containing RTFs at intermediate Ca\(^{2+}\) (Fig. 4C). Mechanistically, each result can be explained by either an actin acetylation- or K328Q-induced loss in Tpm-based
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inhibition of myosin binding.

While K326 acetylation is not disqualified as a modifier of Ca\(^{2+}\) sensitivity, our results suggest that relative to K328, K326 acetylation is, at the very least, significantly less impactful when present in RTFs at the tested amount. This is supported by our previous in vivo results that evaluated the specific effects of K326Q and K328Q Ac-mimetic actin on Drosophila flight ability and IFM morphology (30). Flies that ectopically expressed K328Q actin in IFMs, at roughly the same proportion present in RTFs here, were rendered flightless due to muscle destruction caused by severe, myosin-dependent hypercontraction. On the other hand, while flight ability of K326Q-containing flies was significantly reduced, gross morphological structure of the IFMs was preserved. Therefore, when present at comparable levels in muscle, K328Q elicited a far more severe phenotype than K326Q, which suggests that the relative influence of K328 “pseudo”-acetylation on the regulation of striated muscle contraction exceeds that of K326. Our in vitro study corroborates the in vivo data and reveals a potential molecular mechanism to explain the exacerbated K328Q muscular phenotype. Relative to WT and K326Q, K328 “pseudo”-acetylation of actin increases myosin binding to thin filaments due to elevated Ca\(^{2+}\) sensitivity and a greater loss in Tpm-based inhibition. These effects likely result in dysregulation of force production in vivo, which elicits destruction of IFMs; muscles that are particularly sensitive to perturbation.

Previous work has demonstrated a correlation between myofilament and myofibrillar protein acetylation levels and muscle function (35, 37); however, individual contributions from the various modified proteins to overall behavior remain, for the most part, unknown. One study reported that enzyme-mediated lysine acetylation of striated muscle myosin in vitro increases actomyosin affinity and F-actin sliding velocity, suggesting an acetylation-dependent enhancement in actomyosin cycling (61). In contrast, our data suggest that actin acetylation does not appreciably affect actomyosin activity except when Tpm is present. Recently, we found that introduction of cardiac TnI Ac-mimetic K132Q decreased Ca\(^{2+}\) sensitivity of both RTFs and isolated myofibrils (62). In comparison, K328Q actin increased RTF Ca\(^{2+}\) sensitivity. Future work should focus on how acetylation of other known targets in muscle, e.g. TnT, TnC, titin, etc., individually and in combination, affect actomyosin activity and muscle performance (32).

Given both the Ac-reactivity and functional importance of actin K328 (30, 38), it will be important to determine whether its acetylation status is sensitive to changes in cellular Ac-CoA levels, either physiological (e.g. feeding/fasting) or pathological (e.g. heart failure (63, 64)). Alternatively, increased/decreased KAT or KDAC enzymatic activity could alter levels of the PTM via a residue-specific mechanism. While a number of small-molecule compounds have been shown to affect Ac-CoA tissue levels by inhibiting Ac-CoA carboxylase (65-68), discovery of specific KAT and/or KDAC activators/inhibitors that preferentially target sarcomeric actin, and in particular K326 and/or K328, has been absent.

Ultimately, it will be informative to assess the site-specific stoichiometry of acetylation on actin, both in vitro and in vivo. Preliminary assessments, by targeted parallel reaction monitoring mass spectrometry, are qualitatively consistent with K326 and K328 as highly reactive acetylation sites on actin (Cammarato & Foster, unpublished), as reported previously (30, 38). However, rigorous absolute quantitation at all previously reported acetylation sites on actin is technically challenging owing to the presence of easily-oxidized cysteine and methionine residues flanking many of the acetylation sites (see Table 1). Nevertheless, site-specific actin Ac-peptide assays are in development.

In conclusion, notwithstanding the apparent marginal impact of actin acetylation on global F-actin-Tpm binding affinity and stoichiometry, it reduced Tpm’s ability to block actomyosin interactions and sensitized RTFs bathed in a submaximal, activating amount of Ca\(^{2+}\). Site-specific mimicry of acetylation at the reactive residue, K328 on actin, is sufficient to recapitulate the Ca\(^{2+}\) sensitization. We submit that the physiological acetylation status of K328 and its susceptibility to change, in the context of disease, warrant further scrutiny as the PTM may serve as a potent modulator of striated muscle contraction.
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EXPERIMENTAL PROCEDURES

Molecular modeling

Actin-myosin-Tpm and actin-Tpm structures were generated using Chimera v. 1.9 (69) derived from PDB model #4A7f (41) and 9PDB file provided in Orzechowski et al., 2014 (12).

Drosophila stocks and husbandry

All flies were raised at 25°C on a standard cornmeal-yeast-sucrose-agar medium. The Act88F-Gal4 line was a gift from Dr. Richard M. Cripps (University of New Mexico, Albuquerque, NM), and flies containing one of the UAS-Act57B actin transgenes (UAS-Act57B<sup>GFP,WT</sup>, UAS-Act57B<sup>WT</sup>, UAS-Act57B<sup>K326Q</sup>, or UAS-Act57B<sup>K328Q</sup>) were generated as described previously (30). Virgin Act88F-Gal4 female flies were crossed with males containing one of the UAS-Act57B transgenes to create progeny that overexpressed transgenic Act57B<sup>WT</sup>, Act57B<sup>K326Q</sup>, or Act57B<sup>K328Q</sup> actin specifically in the IFM.

Protein sources, preparation, and quantitation

Lyophilized rabbit skeletal actin was obtained from Cytoskeleton (Cat. #AKL95). Psoas muscle myosin was purified (70) from white New Zealand rabbits provided by Dr. David Kass (Johns Hopkins University). Tissue-purified porcine cardiac Tpm was provided by Dr. Jeff Moore (University of Massachusetts-Lowell) and bovine cardiac isoforms of Tpm and Tn were provided by Dr. Larry Tobacman (University of Illinois at Chicago). Pools containing ectopically-expressed transgenic Act57B<sup>WT</sup>, Act57B<sup>K326Q</sup>, or Act57B<sup>K328Q</sup> actin were purified from Drosophila IFMs according to previously published protocols (22, 49, 54).

In vitro actin acetylation and quantitation of lysine acetylation levels

In vitro actin acetylation was performed as described in Hitchcock-DeGregori, et al. (38). 10 mg/ml globular (G-) actin, re-hydrated as per manufacturer’s instructions in 5 mM Tris-HCl pH 8.0, 0.2 mM CaCl₂, 0.2 mM ATP, 5% (w/v) sucrose and 1% (w/v) dextran, was distributed into 10 µl aliquots, flash-frozen in liquid nitrogen, and stored at -80°C. Thawed G-actin was diluted 10-fold in low-salt buffer (25 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 25 mM imidazole pH 7.2, 10 mM DTT, 1 mM ATP) and polymerized for ~1 h at room temp. Stock acetic anhydride (Sigma-Aldrich Lot # SHBJ2093) was diluted 1:70 (4-fold ratio of total moles of acetic anhydride:total actin lysines) or 1:5600 (0.05 total moles of acetic anhydride:total actin lysines) in 100% acetonitrile and added to 96 µl of 1 mg/ml F-actin via addition of four, 0.3 µl aliquots every 3 min with continuous agitation. Actins were stored at 4°C overnight and dialyzed twice in the cold room against 1 L of buffer (100 or 200 mM KCl, 5 mM MgCl₂, 5 mM Tris-HCl pH 7.5) for at least 4 h to remove unreacted acetic anhydride.

Dialyzed acetylated actin was prepared in 4X LDS sample buffer (Invitrogen Lot #1920134) and ~3 µg was loaded onto a 4-15% gradient SDS-PAGE gel (BIO-RAD Cat. #456-1086). Proteins were transferred to a nitrocellulose membrane, blocked for 1 h in 1:1 mixture of Tris-buffered saline (TBS) with 0.1% Tween 20 (Sigma Lot #SLBD6080V) and blocking buffer (LI-COR #927-40000), and probed with anti-Ac-lysine (Cytoskeleton Cat. #AAC-03) antibodies, diluted 1:750 in TBS, for 1 h at room temp. or overnight in the cold room. Following 4-5 washes in TBS with 0.1% Tween-20 (TBST), Ac-lysine-bound primary antibodies were conjugated to 1:10,000 green secondary antibodies (LI-COR Biosciences) via 1-h incubation at room temp. After numerous TBST washes, membranes were exposed to a 1:7500 diluted anti-actin primary antibody (Proteintech Cat. #20536-1-AP) at room temp. for 1 h, washed, and conjugated to 1:10,000 diluted red secondary antibodies (LI-COR Biosciences). Unconjugated antibodies were washed, and membranes imaged on a LI-COR Odyssey fluorescence imager. Anti-Ac-lysine intensities were normalized to actin and average fold increase in actin-normalized lysine acetylation was determined for 9-11 individual measures.

Sample peptide preparation, chromatography, MS, and data analysis

48 µl of 1 mg/ml F-actin (25 mM KCl, 4 mM MgCl₂, 1 mM EGTA, 25 mM imidazole pH 7.2, 10 mM DTT, 1 mM ATP) were aliquoted into two separate tubes. Each tube was mixed with either
Actin lysine acetylation alters tropomyosin positioning

100% acetonitrile or acetonitrile containing 1:140 (v/v) diluted stock acetic anhydride (Sigma-Aldrich Lot # SHBJ2093) via addition of four, 0.3 µl aliquots every 3 min under constant mixing. Total moles of acetic anhydride added to the latter mixture was four times the number of actin lysines. F-actin samples were left at 4°C overnight, and the following day ~5 µg total protein were dried down, re-dissolved in 50 µl of 20 mM ammonium bicarbonate (pH 8.0), then reduced with 5 µl of 50 mM DTT at 55°C for 1 h and alkylated with 5 µl 50 mM iodoacetamide in the dark for 15 min. Enzymatic cleavage was performed with MS-grade trypsin/lys-C (Promega) at 37°C overnight. Peptides were desalted with stage tips constructed from Empore C18 material (3M Corporation).

Tryptic peptides were subjected to nano-reversed phase high-pressure liquid chromatography (RP-HPLC) coupled to tandem mass spectrometry (MS/MS). Chromatography was conducted on a 75 µm x 250 mm column that was packed in-house into a picofrit self-pack (New Objective) with ReproSil-Pur C18-AQ (3 µm particles, 120 Å pores; Dr. Maisch, GmbH) and eluted with a 120 min linear two-solvent gradient (solvent A: 0.1% (v/v) formic acid (FA; Suprapur, EMD Millipore) with in-house deionized, reverse-osmosed water; solvent B: 0.1% FA in 80% (v/v) acetonitrile (Fisher Scientific) into an Orbitrap Fusion Lumos mass spectrometer equipped with the Easy-IC (internal calibration; Thermo Scientific) option. Data-dependent MS/MS acquisition was employed using a 3 s cycle time between MS scans. The resolution was 120,000 for MS and 30,000 for MS/MS. Signal targets were 2 x 10^6 ions in 60 s for MS and 5 x 10^4 ions in 54 s for MS/MS.

Acquired masses were searched against the RefSeq83 Oryctolagus cuniculus database using Mascot v2.6.2 (Matrix Science), as implemented in Proteome Discoverer 1.4 (Thermo Scientific) with lysine acetylation (+42 Da), deamidation of asparagine and glutamine (+1 Da), and oxidation of methionine (+16 Da) included as variable search parameters. Carbamidomethyl cysteine (+57 Da) was set as a static search parameter. The precursor ion mass tolerance was set to 5 ppm, while 0.02 Da was used for MS/MS. Three missed cleavages were permitted. Data from Proteome Discoverer (1% FDR) were imported into Scaffold Q+ v4.1.0 (Proteome Software) for conversion to mzIdentML and subsequent data inspection in Scaffold PTM v3.3.0 (Proteome Software). Lysine acetylation site confidence was assessed using Scaffold PTM’s implementation of the A-Score (71).

F-actin-Tpm co-sedimentation

Dialyzed control and acetylated F-actin were diluted ~5-fold in buffer that yielded a final composition of 200 mM KCl, 2 mM MgCl2, 10 mM Tris-HCl pH 7.5, and 10 mM DTT. Stock Tpm concentration (65 µM) was determined via Bradford colorimetric assay (Bio-Rad), and 25 µl F-actin (~3.5 µg) were mixed with minute volumes of porcine cardiac Tpm in duplicates at the following Tpm:actin molar ratios: 1:16, 1:15, 1:12, 1:10, 7:1, 1:5, 1:4, 1:3, 1:2, 1:1, 1:5:1, 2:1. Mixtures were placed at room temp. for 30 min, then on ice for a minimum of 2 h and spun-downd in a TLA-100 ultracentrifuge rotor at 100,000 rpm (436,000 x g) for 20 min at 4°C. 12 µl of supernatant were added to 3 µl 4X LDS sample buffer (Invitrogen Lot # 1920134), while 15 µl of 1X sample buffer were added to the pellets. Samples were heated to ~90°C for 3-5 min, centrifuged, loaded on a 4-15% gel (Bio-RAD Cat. # 161-0786) and destained in double distilled water. In addition to assessment of F-actin-Tpm binding at “medium” ionic strength (i.e. 200 mM KCl), co-sedimentation experiments were equivalently performed under low (40 mM KCl, 2 mM MgCl2, 0.8 mM EGTA, 20 mM imidazole, 1 mM Tris-HCl pH 7.5, and 10 mM DTT) and high (500 mM KCl, 2 mM MgCl2, 5 mM Tris-HCl pH 7.5, 10 mM DTT) ionic strengths at 1:3 and 1:4 Tpm:actin molar ratios. KCl concentration was further increased to 820 mM (2 mM MgCl2, 5 mM Tris-HCl pH 7.5, 10 mM DTT) and F-actin-Tpm binding determined at 1:2, 1:1, 1.5:1, and 2:1 Tpm:actin ratios.

Determination of F-actin-Tpm affinity

Coomassie-stained gels were scanned using a LI-COR Odyssey fluorescence imager, and band intensities were quantified using ImageStudio software. Known amounts of Tpm (0.5-6 µg) were used to plot a standard intensity curve to determine
µg of Tpm in pellet and supernatant fractions and converted to moles (67 kDa). Negligible amounts of Tpm were found in pellet fractions in the absence of F-actin (Fig. S5). To determine moles of actin, relative amounts present in pellet and supernatant fractions were calculated by dividing intensities of each band by their sum. The amount of actin in pellet and supernatant fractions was then calculated by multiplying by total actin and converted to moles (42 kDa). Tpm:actin molar ratios were plotted against free Tpm concentrations, and data were fit to the Hill equation ($y = \frac{B_{\text{max}} * x^h}{(K_d + x^h)}$) to derive $B_{\text{max}}$ and $K_d$ of Tpm for control and acetylated actin. Significant differences in fit parameters were assessed via extra sum of squares F-tests, with $p < 0.05$ deemed significant. Percent saturation of F-actin-Tpm binding was defined as the molar ratio of Tpm to actin in the pellet times 700.

F-actin, F-actin-Tpm, and RTF in vitro motility

A portion of dialyzed F-actin was diluted in low-salt buffer to ~1 µM and fluorescently labeled overnight via equimolar addition of Alexa-568 phalloidin (Ph) (ThermoFisher Cat. #A12380). In vitro motility of control and acetylated Alexa-568 Ph-labeled F-actin was performed at myosin concentrations between 12.5 and 100 µg/ml at 30 °C, pH 7.2, and an ionic strength of 37 mM. Briefly, myosin was introduced into a flow cell and allowed 2 min to bind a nitrocellulose-coated cover slip. The surface was blocked with 2 mg/ml BSA, myosin “dead heads” were non-reversibly bound to unlabeled actin filaments, and enzymatically active myosin was bound to < 10 nM Alexa568-Ph-labeled F-actin. Fluorescent actin was excited and imaged using an X-CITE 120 LED lamp and 531/40 filter on an Olympus IX73 microscope. Emitted light was captured at 593/40 and detected on a Hamamatsu Flash 4LT EMCCD camera, operated with HCI imaging software. Videos were converted to multipage TIFs and imported into ImageJ for processing. Duplicate F-actin motility assessments from two separate supra-stoichiometric acetylation reactions, and one sub-stoichiometric acetylation reaction, were obtained in parallel with control F-actins. Videos from 4-8 areas of each flow cell were recorded at 1-10 fps for 20 frames total, and velocities of moving filaments were measured via automated tracking by ImageJ plugin wrmtrack (72). Average velocities and errors from two technical replicate experiments per biological replicate were calculated for filaments classified as movers (see Supporting Information). Total filaments analyzed per myosin concentration ranged between 250 and 2000.

Alexa-568 Ph-labeled F-actin was also incubated with 300 nM bovine cardiac Tpm on ice for at least 30 min prior to F-actin-Tpm motility. Motility buffer also included 150 nM Tpm to maintain Tpm saturation of F-actin. Average velocities and percent filaments moving of control and acetylated F-actin-Tpm samples were normalized to respective F-actin velocities and percent filaments moving at each myosin concentration. F-actin normalized F-actin-Tpm motility parameters were subtracted from one and multiplied by 100 to determine the percent of Tpm-based inhibition at each myosin concentration. The effect of acetylation on Tpm-based inhibition was assessed via two-way ANOVAs with $p < 0.05$ considered significant.
DATA AVAILABILITY
All data, associated protocols, methods, and sources of materials are available in the main text or in the Supporting Information. Additionally, the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (73) partner repository with the dataset identifier PXD020732 and 10.6019/PXD020732.

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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
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### Abbreviations

| Abbreviation (in order of appearance) | Full Meaning |
|---------------------------------------|--------------|
| PTM                                   | Post-translational modification |
| Tpm                                   | Tropomyosin |
| Tn                                    | Troponin |
| TnI                                   | Inhibitory subunit of troponin |
| TnT                                   | Tropomyosin-binding subunit of troponin |
| TnC                                   | Calcium-binding subunit of troponin |
| B-state                               | Blocking positional state of tropomyosin |
| C-state                               | Closed positional state of tropomyosin |
| M-state                               | Myosin-induced positional state of tropomyosin |
| A-state                               | “Apo-“ positional state of tropomyosin |
| Indirect flight muscle                | IFM |
| KAT                                   | Lysine acetylase |
| KDAC                                  | Lysine deacetylase |
| B<sub>max</sub>                       | Maximum binding |
| K<sub>d</sub>                         | Binding affinity |
| KCl                                   | Potassium chloride |
| Act88F                                | *Drosophila* skeletal actin |
| ACTA                                  | Vertebrate skeletal actin |
| Act57B                                | *Drosophila* cardiac actin |
| ACTC                                  | Vertebrate cardiac actin |
| G-                                    | Globular |
| HCl                                   | Hydrochloric acid |
| CaCl<sub>2</sub>                      | Calcium chloride |
| MgCl<sub>2</sub>                      | Magnesium chloride |
| LDS                                   | Lithium dodecyl sulfate |
| TBS                                   | Tris-buffered saline |
| TBST                                  | Tris-buffered saline with 0.1% Tween-20 |
| Ph                                    | Phalloidin |
Table Legend

Table 1. Chemically acetylated lysine residues on actin. Actin was acetylated with an 80:1 molar ratio of acetic anhydride:actin, digested with trypsin and analyzed by MS as described in Experimental Procedures. Acetylated peptides were identified by a mass search of in silico-trypsinized rabbit skeletal actin (UniProtKB- P68135), allowing for variable modification of lysine residues with a mass shift of 42 kDa (71). Annotated spectra associated with each peptide can be found in the online Supporting Information (Fig. S6).
Figure Legends.

**Fig. 1.** Actin acetylation does not significantly alter myosin-driven F-actin sliding velocity.
A) Proposed K328-E286 (blue-red respectively) actin-myosin (grey-tan respectively) salt bridge established during strong binding (PDB # 4A7f) (41). B) Western blots of actin treated with supra-stoichiometric acetic anhydride diluted in acetonitrile (acetylated) revealed increased lysine acetylation relative to actin resuspended in acetonitrile only (control). Blots were probed with anti-actin (top) and anti-Ac-lysine (bottom; Anti-Ac-K) antibodies. C) Anti-Ac-K intensities in B) were normalized to corresponding anti-actin signals. Actin-normalized lysine acetylation increased ~290-fold (291 ± 37) relative to control. D) *In vitro* motility of Alexa-568 Ph-labeled control (black) and acetylated (grey) F-actin at varying myosin concentrations. Average velocities (VelocityAvg.) were not significantly different, suggesting no change in actomyosin cross-bridge cycling due to actin acetylation (two-way ANOVA; n = 4).

**Fig. 2.** Actin acetylation does not disrupt global F-actin-Tpm binding.
A) Purported contacts between K326/K328 (blue) on actin (grey) and negatively charged Tpm (purple) residues (red) in pseudorepeats 4, 5, and 6 (from bottom to top) (12). B) F-actin-Tpm co-sedimentation data were fit to the Hill equation \( y = B_{\text{max}} x^h/(K_d^h + x^h) \), and no significant differences were found in \( B_{\text{max}} \) or \( K_d \) of Tpm for control (black; \( B_{\text{max}} = 0.15 \pm 0.009; K_d = 0.21 \pm 0.03 \mu M \) respectively) vs. acetylated (grey; \( B_{\text{max}} = 0.14 \pm 0.013; K_d = 0.15 \pm 0.04 \mu M \) respectively) F-actin. C) Saturating amounts of Tpm were mixed with control (black) or acetylated (grey) F-actin at 40, 200, 500, and 820 mM [KCl] and pelleted. At 40 mM, percent Tpm saturation of control (112 ± 6.3%) vs. acetylated (113 ± 2.2%) F-actin was equivalent. Increasing [KCl] to 200 mM similarly decreased Tpm binding to control (85.4 ± 6.85%) and acetylated (70.4 ± 4.68%) F-actin, which was nearly fully ablated by 500 mM.

**Fig. 3.** Actin acetylation reduces Tpm-based inhibition of actomyosin binding.
A) Control (black) and B) acetylated (grey) F-actin (solid) and F-actin-Tpm (checkered) velocities significantly increased as a function of myosin concentration, while Tpm addition significantly decreased velocities (two-way ANOVA; \( p < 0.0001; n = 4 \)). C) Percent decrease in control (black) and acetylated (grey) F-actin velocities observed as a result of Tpm addition revealed that acetylation had no significant effect on Tpm-mediated reduction of velocity (two-way ANOVA; \( n = 4 \)). The effects of myosin concentration and Tpm on percent moving of D) control (black) and E) acetylated (grey) F-actin (solid) and F-actin-Tpm (checkered) mirrored those on velocity, while F) actin acetylation (grey) significantly decreased Tpm-based inhibition of filament motion relative to control (black) (two-way ANOVA; \( p < 0.0001; n = 17-22 \)).

**Fig. 4.** Reduced Tpm-based inhibition persists in acetylated actin-containing RTFs.
A) Representative images illustrating immeasurable (pCa 9) and equivalent (pCa 4) movement of control and acetylated actin-containing RTFs by overlaying the first frame of a 17-20 s movie (white) with a summative image of total motion (yellow). Scale bar = 7 µm. B) Percent filaments moving (left; \( n = 9 \)) and average velocities (right; \( n = 2 \)) of control (black; 40.1 ± 3.9% and 1.19 ± 0.21 µm/s respectively) and acetylated (grey; 40.5 ± 3.4% and 1.11 ± 0.17 µm/s respectively) RTFs at pCa 4 were indistinguishable (two-tailed \( t \)-test). C) Percent filaments moving (left; \( n = 7 \)) and average velocities (right; \( n = 2 \)) of acetylated (24.75 ± 1.63% and 0.27 ± 0.01 µm/s respectively) RTFs were significantly greater than control (20.11 ± 1.45% and 0.18 ± 0.02 µm/s respectively) at pCa 6.5 (two-tailed \( t \)-test; \( ^* p < 0.05 \)).

**Fig. 5.** K328 “pseudo”-acetylation enhances RTF Ca\(^{2+}\) sensitivity.
A) Treatment of actin with a sub-stoichiometric amount of acetic anhydride (checkered grey) significantly increased percent of actin-Tpm filaments moving relative to control (black) (two-way ANOVA; \( p < 0.001 \); \( n = 9-12 \)), while sub-stoichiometric-treated F-actin-Tpm movement was not statistically different from supra-stoichiometric-treated (grey) (two-way ANOVA, \( n = 10-12 \)). B) and C) Velocity-normalized plots of Ca\(^{2+}\)-dependent activation of K326Q- and K328Q-containing RTFs relative to respective WT controls. K326Q and K328Q maximum velocities (\( V_{\text{max}} = 4.1 \pm 0.1 \) µm/s and 3.3 \( \pm 0.06 \) µm/s) did not significantly differ from respective internal WT controls (\( V_{\text{max}} = 4.2 \pm 0.09 \) µm/s and 3.3 \( \pm 0.06 \) µm/s respectively). (B) Ca\(^{2+}\)-dependent activation of K326Q-containing RTFs was equivalent to WT control actin-containing RTFs as revealed by no significant differences in Ca\(^{2+}\) sensitivity (\([\text{Ca}^{2+}]_{50} = 0.43 \pm 0.029 \) µM vs. 0.42 \( \pm 0.027 \) µM respectively), or cooperativity (\( h = 1.8 \pm 0.25 \) vs. 1.7 \( \pm 0.21 \) respectively). (C) While there was no significant change in cooperativity of K328Q-containing RTFs (\( h = 1.8 \pm 0.21 \)) relative to WT control (\( h = 2.2 \pm 0.22 \)), K328Q-containing RTF Ca\(^{2+}\) sensitivity (\([\text{Ca}^{2+}]_{50} = 0.56 \pm 0.032 \) µM) was significantly increased relative to WT control (\([\text{Ca}^{2+}]_{50} = 0.87 \pm 0.044 \) µM) (\( p < 0.0001 \); \( n = 4 \)).
| Entry | Acetylated Peptide Sequence | Modifications | Protein Accessions | Protein Name | Acetylation Site #a | Peptide Identification Probability | Acetyl Site Localization Probability b | Mascot Best Ion Score c | Mascot Identity Score d | Observed Mass | Actual Mass | Charge | Delta PPM |
|-------|-----------------------------|---------------|-------------------|--------------|---------------------|-----------------------------------|------------------------------------|---------------------|-------------------|----------------|------------|---------|----------|
| 1     | (K)DSYVGDEAQSm(R)          | Acetyl (+42)  | XP_002722940.1, XP_002718044.1, NP_001095153.1 | Actin, Ca & Cy | K61                 | 100%                              | 100%                               | 79.84               | 27.19            | 698.8216      | 1,395.63   | 2       | 1.354    |
| 2     | (R)VAPEHPTLLEAPNPKAn(R)    | Acetyl (+42)  | XP_002722940.1, XP_002718044.1 | Actin, Ca   | K113                | 100%                              | 100%                               | 78.04               | 31.45            | 780.4176      | 2,338.23   | 3       | 1.127    |
| 3     | (R)DILTDYbLLe(R)           | Oxidation (+16), Acetyl (+42) | XP_002722940.1, XP_002718044.1, NP_001095153.1 | Actin, Ca & Cy | K191                | 100%                              | 100%                               | 51.8                | 30.77            | 551.6243      | 1,651.85   | 3       | 1.044    |
| 4     | (R)DILTDYbLLe(As)          | Oxidation (+16), Acetyl (+42) | XP_002722940.1, XP_002718044.1, NP_001095153.1 | Actin, Ca & Cy | K191                | 100%                              | 100%                               | 62.48               | 30.76            | 834.9309      | 1,667.85   | 2       | 1.676    |
| 5     | (K)eLYVALDfEFQMaTASSSSSeKcS | Acetyl (+42), Carbamidomethyl (+57) | XP_002722940.1, XP_002718044.1 | Actin, Ca & Cy | K215                | 100%                              | 100%                               | 109.05              | 31.86            | 945.7747      | 2,834.30   | 3       | 1.139    |

### Table 1

a Site numbering according to the sequence, *in vivo*, which excludes the first two amino acids that are cleaved post-translationally.
b Position of Ac moiety assessed using the A-Score algorithm of Beausoleil et al. (71).
c Ion Score = -10log(probability that match is random)
d Identity Score is the score greater than which peptide identity has p<0.05.
Figure 1
Figure 2
Figure 3

A: Velocity Avg.
- Control F-actin
- Control F-actin + Tpm

B: Velocity Avg.
- Acetylated F-actin
- Acetylated F-actin + Tpm

C: Extent of Tpm inhibition on velocity
- Control
- Acetylated

D: Motile filaments
- Control F-actin
- Control F-actin + Tpm

E: Motile filaments
- Acetylated F-actin
- Acetylated F-actin + Tpm

F: Extent of Tpm inhibition on movement
- Control
- Acetylated
Figure 4
Figure 5

A

% Filaments Moving

control
supra-treated
control vs. supra-treated
myosin: p=0.001
supra vs. sub-treated
myosin: p=0.001

[Myosin] (µg/ml)

12.5  25.0  50.0  100.0

B

Norm. Vel. (V/V_{max})

WT
K326Q

[Ca^{2+}] (M)

10^{-9}  10^{-8}  10^{-7}  10^{-6}  10^{-5}  10^{-4}

C

Norm. Vel. (V/V_{max})

WT
K326Q

[Ca^{2+}] (M)

10^{-9}  10^{-8}  10^{-7}  10^{-6}  10^{-5}  10^{-4}
Lysine acetylation of F-actin decreases tropomyosin-based inhibition of actomyosin activity
William Schmidt, Aditi Madan, D. Brian Foster and Anthony Cammarato

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