**Cardiac actin changes in the actomyosin interface have
different effects on myosin duty ratio**

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**Cardiac actin changes in the actomyosin interface have different
effects on myosin duty ratio**

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Abstract

Hypertrophic cardiomyopathy (HCM) is an inherited cardiovascular disease (CD) that commonly causes an increased size of cardiomyocytes in the left ventricle. The proteins myosin and actin interact in the myocardium to produce contraction through the actomyosin ATPase cycle. The duty ratio (r) of myosin is the proportion of the actomyosin ATPase cycle that myosin is bound to actin and does work. A common hypothesis is that HCM mutations increase contraction in cardiac sarcomeres; however, the available data is not clear on this connection. Based on previous work with human α -cardiac actin (ACTC), we hypothesize that HCM-linked ACTC variants with alterations near the myosin binding site have an increased r , producing more force. Myosin duty ratios using human ACTC variant proteins were calculated with myosin ATPase activity and *in vitro* motility data. We found no consistent changes in the duty ratio of the ACTC variants, suggesting that other factors are involved in the development of HCM when ACTC variants are present.

Keywords: hypertrophic cardiomyopathy, cardiac actin, mutations, actomyosin, duty ratio

Introduction

Cardiovascular disease (CD) is the most costly global disorder and has become the greatest cause of mortality in the world, being responsible for one third of deaths worldwide with immense economic costs (“WHO | Cardiovascular diseases (CVDs)” 2016). In Canada alone, where there are more than 35 million people, over \$20 billion is spent annually to treat CD (Tarride et al. 2009). Among the many forms of CD, hypertrophic cardiomyopathy (HCM) is an inherited disease that, among a heterogeneous spectrum of abnormalities (Pandian et al. 2015), commonly results in the overgrowth of the left ventricle and is a leading cause of sudden cardiac death in individuals under the age of 30 (Seidman and Seidman 2001, Gajewski and Saul 2010, Maron et al. 2014).

The functional units of the heart muscle are sarcomeres which contain actin and myosin proteins. These interact together in a power stroke, shortening the sarcomeres, leading to muscle contraction. The power stroke requires the hydrolysis of ATP by myosin when bound to actin (Fox, 2012). The force produced by myosin and actin in the sarcomere is termed the ensemble force and can be calculated with the equation:

$$F_{EN} = N_t \cdot f_i \cdot r, \quad (\text{Eq 1})$$

where N_t is the number of individual actomyosin interactions, f_i is the force per actomyosin interaction, and r is the duty ratio of myosin (Sommese et al. 2013). The myosin duty ratio is the proportion of the actomyosin ATPase cycle that myosin is bound to actin and can be calculated using the equation:

$$r = (k_{cat} \cdot \delta) / v_o \quad (\text{Eq 2})$$

where k_{cat} is the k_{cat} of myosin’s ATPase activity, v_o is the velocity of the enzyme, and δ is the step size of myosin (Howard 1997).

The symptoms of HCM patients commonly include hyperdynamic systolic function (Pandian et al. 2015), suggesting that sarcomere protein changes in HCM hearts result in hypercontractility. However, current data does not sufficiently connect HCM-causing mutations with hypercontractile sarcomeres (discussed in (Spudich 2014)). Nevertheless, changes in the duty ratio of myosin will reflect changes in the contraction of HCM-affected hearts, with higher duty ratios associated with hypercontractility.

HCM has been associated with 12 different α -cardiac actin (ACTC) gene mutations. Four ACTC variants found in HCM patients possess changes in or near the actomyosin-binding site: H88Y, F90 Δ , R95C, and E99K (**Figure 1A**) (Olson et al. 2000, Morita et al. 2008, Kaski et al. 2009, Behrmann et al. 2012). We previously examined the connection between HCM-related human ACTC mutations and altered actomyosin interactions with 8 variants of ACTC, including the E99K variant located in the actomyosin-binding site (Dahari and Dawson 2015). While the E99K ACTC protein resulted in a higher duty ratio, we found that the other variants resulted in duty ratios similar to wild-type ACTC protein. We hypothesized that the position of the amino acid change on ACTC resulting from mutation impacts proteins binding at that site. For example, the E99K substitution is located in the actomyosin binding site (Behrmann et al. 2012) and will affect myosin function. R95 on ACTC may form a salt bridge with E576 and hydrogen bonds with three other residues on myosin (Lorenz and Holmes 2010). With the loss of these interactions, R95C ACTC might exhibit similar defects to those of E99K-ACTC. With H88Y ACTC, the affected residue is on the same alpha helix in ACTC as R95 and E99. While more distal from the actomyosin interface, an amino acid substitution may interfere with the packing of residues along the helix, thereby indirectly inhibiting actomyosin interactions. Finally, deletion mutants in actin are very rare, and the deletion of F90 in the helix containing R95 and

E99 might alter the register and position of those amino acids, possibly disrupting interactions in the actomyosin interface, resulting effects on actomyosin activity.

To test this hypothesis, we produced four HCM-related human ACTC variant proteins with changes in or near the actomyosin binding site, including the E99K variant for comparison. We calculated the duty ratios using myosin ATPase activity and *in vitro* motility assays, with the expected result that all variants in the actomyosin binding site will exhibit increased myosin duty ratios. Our work is the first biochemical analysis of the three subdomain-1 ACTC variant proteins H88Y, F90Δ, and R95C. Contrary to our expected results, we found that only E99K-ACTC produced an increase in the duty ratio of myosin, suggesting that multiple factors play a role in the development of HCM in the presence of other subdomain-1 ACTC variants.

Materials and methods

Reagents and Basic Protocols

All reagents were obtained from Thermo Fisher Scientific or Sigma-Aldrich, unless stated otherwise. Recombinant purified ACTC protein concentrations were determined using Bio-Rad protein assay dye and skeletal actin standards following the manufacturer's protocol (Hercules, CA). 10% polyacrylamide resolving gels were used to conduct SDS-PAGE with a 5% stacking gel. Samples were mixed with 2x Laemmli buffer at a 1:2 ratio of buffer to sample (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 1.3 M βME and 0.1% bromophenol blue) (Laemmli 1970). Gels were run for 45 minutes at 180 mV with a 1X running buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), and stained with Coomassie Stain and destained in 40% methanol, 10% acetic acid.

Protein Purification

Pure myosin and heavy meromyosin (HMM) was obtained from rabbit *soleus* muscle (Margossian and Lowey 1982). The myosin obtained from the *soleus* muscle of rabbits is a combination of >90% Type I (slow myosin; low ATPase activity) and <10% Type II (fast myosin; high ATPase activity)(Billeter et al. 1980, Reiser et al. 1985). This is comparable to the myosin found in human cardiac ventricles which is a combination of <10% α -myosin heavy chain (fast) and >90% β -myosin heavy chain (slow)(Reiser et al. 2001, Narolska et al. 2005).

α -cardiac actin protein was prepared from the left ventricular muscle of bovine hearts obtained from the Animal and Poultry Science Department (Guelph, ON) (Spudich and Watt 1971). Bovine α -cardiac actin was used as a control as it shares 100% sequence identity to human α -cardiac actin protein. Bovine actin concentrations were determined by measuring absorbance at 290 nm using an extinction coefficient of $0.62 \text{ mL} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$. Other protein concentrations were determined with Bio-Rad protein dye reagent employing bovine actin as the standard.

Recombinant gelsolin and His-tagged G4-6 proteins were expressed and purified from *Escherichia coli* following established protocols (Morrison and Dawson 2007, Ohki et al. 2009).

Recombinant human ACTC variant proteins were expressed in Sf21 cells with recombinant baculoviruses (Mundia et al. 2012). To purify the recombinant ACTC proteins, we adapted the affinity method of Ohki *et al.* (2009), binding actin proteins in cell lysates to His-tagged G4-6 in the presence of calcium and eluting the ACTC proteins in an EGTA-containing buffer. Cells from a 500 mL culture were first harvested by centrifugation and lysed in Buffer A (10 mM Tris, pH 8.0, 5 mM CaCl_2 , 0.2 mM PMSF, 0.01 mg/mL leupeptin, 1 mM ATP, 2 mM β -mercaptoethanol) by vortexing with glass beads (BioSpec Products) in a 50 mL conical tube for 6 cycles of 1 minute vortexing followed by 4 minutes on ice. 10 mg of His-G4-6 was then added

119 to the lysate and mixed at 4°C overnight. After centrifugation at 25,000 rpm in a TA 25.50 rotor
120 (Beckman) for 30 minutes, the supernatant was recirculated through a 5 mL Ni-NTA column
121 (Qiagen) in a closed loop for 1.5 hours. The column was then washed with 30 mL of Buffer A
122 containing 50 mM KCl followed by 40 mL of Buffer A containing 50 mM KCl and 20 mM
123 imidazole. A 1 mL HiTrap DEAE Sepharose FF column (GE Healthcare Life Science)
124 equilibrated in 10 mM Tris, pH 8.0, 0.2 mM CaCl₂, 1 mM ATP, 0.125 mM PMSF, 0.2 mM β-
125 mercaptoethanol was attached to the bottom of the Ni-NTA column. 40 mL ACTC release buffer
126 (10 mM Tris, pH 8.0, 50 mM KCl, 0.2 mM EGTA, 0.2 mM PMSF, 0.5 mM MgCl₂, 0.2 mM
127 ATP, 2 mM β-mercaptoethanol) was applied to release the ACTC protein from the His-tagged
128 G4-6 bound on the Ni-NTA column. The released ACTC protein then bound to the DEAE
129 column. After disconnecting the DEAE column, the His-tagged G4-6 protein was eluted from the
130 Ni-NTA column in 15 mL G4-6 elution buffer (10 mM Tris, pH 8.0, 50 mM KCl, 0.2 M
131 imidazole, pH 8.0, 5 mM CaCl₂, 1 mM PMSF, 1 mM ATP, 2 mM β-mercaptoethanol). ACTC
132 was eluted from the DEAE column in ACTC elution buffer (10 mM Tris, pH 8.0, 0.2 mM CaCl₂,
133 0.2 mM ATP, 0.2 mM PMSF, 0.2 mM β-mercaptoethanol, 0.3 M KCl). ACTC-containing
134 fractions were dialyzed into G-buffer (2 mM Tris pH 8, 0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM β-
135 mercaptoethanol, 0.002% NaN₃). Proteins were kept on ice and used within one week of
136 preparation. 10% SDS-PAGE was used to verify the presence of pure ACTC and the progress of
137 purification. The average ACTC protein yields per 500 mL cell culture were as follows:
138 recombinant wild type (WTrec), 2.4 ± 0.2 mg (n=22); H88Y ACTC, 4.3 ± 0.4 mg (n=3); F90Δ
139 ACTC, 2.4 ± 0.1 mg (n=3); R95C ACTC, 3.1 ± 0.3 mg (n=14); E99K ACTC, 2.3 ± 0.3 mg
140 (n=6).

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ATPase Assay

The myosin ATPase activity assay used was a colorimetric assay based on the production of a blue byproduct between released P_i and molybdate, modified from Trybus (2000) for a 96-well format. ACTC was polymerized in assay buffer (AB) (75 mM KCl, 10 mM imidazole, pH 7, 10 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM NaN_3), in the presence of a 1:1000 molar ratio of gelsolin to ACTC, and 2 mM Mg-ATP. Gelsolin is added to the ATPase assay to reduce viscosity of high F-actin concentrations, increasing the accuracy in the experiment (Sommese et al. 2013). Samples of varying actin concentrations were produced through serial dilutions in AB containing 2 mM Mg-ATP and kept on ice until they were added to the reaction.

The ATPase assay was performed using two 96-well plates at 22°C. The reaction plate contained concentrations of H_2PO_4 ranging from 0.05 mM to 2 mM for a standard curve and triplicate 100 μ L samples of each ACTC concentration. Rabbit *soleus* muscle myosin was prepared in 1X AB without ATP to a final concentration of 0.125 mg/mL. 100 μ L of myosin was added to each actin sample well to begin the ATPase reaction (final concentration of 0.063 mg/mL or 0.13 μ mol/L). At 10 minute intervals for 40 min, 45 μ L of each reaction mixture was removed and mixed with 45 μ L stop solution (60 mM EDTA pH 6.5, 6.6% SDS) in wells of the stop plate. Over the course of the assay, the ATPase rate was linear for all ATC variants (data not shown). The standard samples were also mixed with 45 μ L stop solution.

After 40 min, 200 μ L of a molybdate colouring solution (0.25M 2% ammonium molybdate, 5 mg/ml ferrous sulfate) were added to the wells of the stop plate and the plate was then read at 750 nm in a microplate reader. The standard curve was used to calculate the rate of inorganic phosphate release in each assay. The average of the triplicate ATPase activities for each concentration of actin was plotted against the concentration of actin in the assay and fit to

the Michaelis-Menten curve using Prism software (GraphPad, La Jolla, CA). k_{cat} was calculated by determining the myosin concentration using a myosin heavy chain mass of 486 kDa. Statistical analysis was accomplished using SPSS (IBM, Armonk, North Castle, NY).

***In Vitro* Motility Assays**

The *in vitro* motility assay was adapted from Toyoshima (1987) and performed at 22°C. Active HMM was obtained by "deadheading" HMM with F-actin in the absence of ATP, co-sedimentation of the acto-HMM complex, and release of active HMM in the presence of 2 mM ATP. Flow cells were produced with nitrocellulose-coated coverslips and double-sided tape on microscope slides where proteins were flowed in and incubated for 2 minutes for protein binding and buffer exchanges. Deadheaded HMM diluted to 0.125 mg/mL in assay buffer was injected first, followed by bovine serum albumin (BSA) diluted to 1 mg/mL in assay buffer to block the nitrocellulose coverslip. The flow cell was then washed with assay buffer, and rhodamine phalloidin-labelled ACTC protein variants (naked and/or regulated thin filaments) diluted to ~0.1 µM were bound to HMM in the nitrocellulose coverslip in the absence of ATP.

The flow cell was then washed with assay buffer prior to the addition of motility buffer (MB; 25 mM KCl, 25 mM imidazole, pH 7.5, 10 mM DTT, 4 mM MgCl₂, 1 mM EGTA, and 2 mM ATP) with an oxygen-scavenging system (25 ug/mL glucose oxidase, 45 ug/mL catalase, and 1% glucose) to prevent photobleaching. The flow cell was then inverted onto a Zeiss Axiovert 200M (Zeiss, Jena, Germany) microscope and actin proteins were visualized at 100x oil-immersed magnification using the Texas Red (510 nm) filter. The movement of filaments was captured using a digital CCD ORCA-R2 C10600 camera (Hamamatsu, Middlesex, NJ)

controlled by Volocity 6.3 software (Perkin Elmer, Waltham, MA) at a frame rate of 2.48 frames/second. Three movies at different locations on the nitrocellulose coverslip were captured. The movement of ACTC filaments was tracked using a protocol adapted from Fraser and Marston (1995). 14 to 20 single ACTC filaments moving in a linear pattern for at least 6 frames were selected in each movie. Each filament's distance moved was tracked using Image J 1.47v and recorded on a customized Excel spreadsheet containing input of individual filament coordinates, number of pixels moved per frame, frame rate displacement, and individual filament velocities. The calibration of 89.5 nm/pixel was used to determine the total distance moved by each filament.

Results

ACTC Purification

Previously, we used DNase-I affinity chromatography to purify recombinant human ACTC proteins from insect cells infected with recombinant baculoviruses (Yates et al. 2009, Mundia et al. 2012, Dahari and Dawson 2015). Here, we used recombinant His-tagged gelsolin segments 4-6 (G4-6) to purify the human ACTC proteins in a calcium-dependent manner (Ohki et al. 2009) using a combination of Ni-NTA columns for purification and DEAE columns for actin concentration. Where DNase-I affinity chromatography requires slower-flowing resin and harsh elution conditions containing 50% formamide, the method employed here uses commercially available fast-flow resin and mild buffer conditions, resulting in 2-4 mg of protein from a 500 mL insect cell culture (**Figure 1B**).

Comparison of Tissue Purified and Recombinant ACTC

To ensure that recombinant ACTC proteins purified with His-G4-6 protein possess the same properties as tissue-purified cardiac actin, we compared actin-activated myosin ATPase activity using bovine cardiac actin or recombinant WT ACTC (WTrec). The resulting Michaelis-Menten curves were similar (**Figure 2 inset**). Bovine cardiac actin produced a myosin ATPase $V_{\max}$ of $14.9 \pm 1.0 \mu\text{M P}_i/\text{min}$ and K_M of $1.2 \pm 0.3 \mu\text{M}$, while WTrec ACTC had a $V_{\max}$ of $14.2 \pm 1.0 \mu\text{M P}_i/\text{min}$ and a K_M of $1.0 \pm 0.4 \mu\text{M}$.

The k_{cat} and Duty Ratio of ACTC Variants

Michaelis-Menten curves for the myosin ATPase activity of each ACTC variant were generated (**Figure 2A**) and their $V_{\max}$ and K_M values determined by fitting the curves to the Michaelis-Menten equation. The $V_{\max}$ for each variant was used to determine the k_{cat} of the reaction given the amount of myosin employed (**Table 1**). No significant differences between WTrec activities were seen compared to E99K and H88Y variants; there were significant differences in comparing WTrec to F90 Δ and R95C.

In vitro motility assays determined the sliding velocity v_o of each ACTC variant over a bed of HMM (**Figure 2B**). There was a significant decrease in v_o between WTrec ($v_o = 1.99 \mu\text{m/sec}$) and E99K ($v_o = 1.25 \mu\text{m/sec}$), consistent with previous studies (Debold et al. 2010, Dahari and Dawson 2015). We also observed a significant increase between WTrec and F90 Δ ($v_o = 2.89 \mu\text{m/sec}$).

Assuming a fixed step size of 5 nm for myosin, we then calculated the duty ratio of each ACTC variant, r , and the relative myosin duty ratio with respect to WTrec ACTC, r_{rel} (**Table 1**). E99K had an increased relative duty ratio of 1.36, consistent with our previous work (Dahari and

Dawson 2015). Conversely, F90Δ, and R95C had lower relative duty ratios of 0.43 and 0.71, respectively, while H88Y had a duty ratio similar to WTrec.

Discussion

Previously, we characterized the intrinsic properties of several human ACTC variant proteins and concluded that changes in subdomains-1 and -4 of actin impact protein-protein interactions (Mundia et al. 2012). We then determined the myosin duty ratio for a number of ACTC variants located along the surface of actin, including E99K ACTC, concluding that different molecular mechanisms exist for ACTC variants leading to HCM development (Dahari and Dawson 2015). In the current work, we focused specifically on the actomyosin-binding site; hypothesizing that ACTC variants in this region follow a similar dysfunctional pattern.

To test our hypothesis, we first purified recombinant human ACTC variant proteins employing His-tagged G4-6 affinity chromatography (Ohki et al. 2009). We showed that tissue-purified bovine ACTC protein and human WTrec protein did not exhibit any statistically significant differences in myosin ATPase assays, indicating that the His-tagged G4-6 purification method does not affect the activity of the resulting proteins.

We predicted that variants in the actomyosin binding site would follow the pattern of the well-studied E99K ACTC variant, exhibiting an increase in myosin duty ratio; however, our data from ATPase and *in vitro* motility assays showed variability between the actomyosin site of other ACTC variants. In ATPase assays, E99K and H88Y ACTC variants exhibited activities similar to WTrec, while R95C and F90Δ had lower ATPase activities. In the *in vitro* motility assays, E99K ACTC had lower sliding velocities, while R95C and F90Δ variants exhibited higher velocities. The H88Y ACTC had a similar velocity to WTrec. Taken together, the

calculated duty ratios based on a 5 nm myosin step size resulted in a duty ratio relative to WTrec that was higher for E99K ACTC, consistent with our previous work (Dahari and Dawson 2015), lower for R95C and F90Δ ACTC and similar for H88Y ACTC.

The hypothesis that a change in the same binding site will result in the same molecular dysfunction does not account for the nature of that change. The E99K substitution results in a charge-reversal at a site that is predicted to participate in a salt bridge with a counterpart amino acid on myosin (Lorenz and Holmes 2010, Behrmann et al. 2012). Rather than form a salt bridge, the charge reversal would result in charge repulsion at this site. H88Y is farther from the predicted actomyosin atomic interactions and is a milder substitution. It is therefore unsurprising that this ACTC variant exhibits properties similar to WTrec. The R95C change results in a loss of charge in a potential salt bridge with myosin, similar to E99K, but it results in the opposite molecular effects. Interestingly, the complete deletion of a codon encoding amino acid 90 in ACTC might result in a change in the local structure and arrangement of amino acids in the actomyosin binding site and results in a similar molecular dysfunction to R95C ACTC.

Despite being in the same molecular location on the actin molecule, the ACTC variants studied here found in patients with HCM show different effects on actomyosin duty ratio. While duty ratio is an important part of the overall contributors to cardiac contractility, ACTC variants might also impact the step size (δ) or the intrinsic force (f) of the actomyosin interaction (Spudich 2014), resulting in changes in force production. To determine if these other parameters are altered, single-molecule laser-trapping assays could be employed.

Our work benefits from being free of the complications of the regulatory machinery found in cells; at the same time, those forms of regulation can compensate for changes in the core force production between actin and myosin. Our approach starts with the core interactions;

to gain a fuller picture, we also need to examine added layers of biological complexity involving the products of other genes that are mutated in patients suffering HCM (Maron 2013), such as examining regulated thin filaments *in vitro* or reconstituted thin filaments using sinusoidal analysis (Bai et al. 2014, 2015). Integration of data from several levels of cardiac function regulation will connect molecular defects to HCM development. These connections will allow us to target specific changes in individual patients, tailoring treatments and leading to longer and better lives.

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Author Contributions

J.D. provided lab space, supervision, supplies, preliminary research and wrote the manuscript. H.L. provided v_o from *in vitro* motility assays with ACTC variants used in the duty ratio calculations. H.L. produced the baculoviruses containing *ACTC* mutants and infected the insect cells with them. M.A. developed baculoviruses and completed preliminary datasets. M.H. performed ATPase assays.

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Tables

Table 1: The relative duty ratio of myosin in the presence of ACTC variant proteins. Duty ratios were determined using data from myosin ATPase assays and *in vitro* motility (IVM) data. V_{max} , K_M , and v_o data are presented as the mean $\pm$ standard error of the mean (SEM). Myosin ATPase assays were performed in triplicate for two biological replicates. One biological replicate was employed for E99K ACTC. For IVM assays, measurements were taken from 3 biological replicates (WTrec, H88Y, R95C) or 2 biological replicates (F90 Δ , E99K).

Variant	Myosin ATPase assays				IVM			
	V_{max} (μ M P _i /min)	k_{cat} (min ⁻¹)	K_M (μ M)	N	v_o (μ m/sec)	N (filaments (assays))	Duty ratio r	Relative duty ratio r_{rel}
WTrec	14.2 $\pm$ 1.0	55.13	1.9 $\pm$ 0.4	5	1.99 $\pm$ 0.02	180 (9)	0.14	1.00
H88Y	12.7 $\pm$ 0.5	49.42	1.5 $\pm$ 0.2	6	1.84 $\pm$ 0.03	180 (9)	0.13	0.97
R95C	11.2 $\pm$ 1.3*	43.46	3.5 $\pm$ 0.9	5	2.23 $\pm$ 0.03	218 (11)	0.10	0.71
F90 Δ	8.8 $\pm$ 0.4*	34.40	1.1 $\pm$ 0.2	6	2.89 $\pm$ 0.06 [†]	120 (6)	0.06	0.43
E99K	12.1 $\pm$ 0.7	47.08	1.5 $\pm$ 0.3	3	1.25 $\pm$ 0.02 [†]	89 (5)	0.19	1.36

* Statistically significant difference ($p < 0.05$) using a *post hoc* Dunnett's test.

[†] Statistically significant difference ($p < 0.05$) using a Kruskal-Wallis test.

Figure captions

Figure 1: (A) A ribbon model of actin (PDB 1ATN) visualized using PyMol showing the locations of HCM-related amino acid changes in the predicted myosin binding site. Bound ATP is shown as sticks. The amino acid numbers listed are based on the sequence of actin following posttranslational modification of the N-terminus removing the first two amino acids of the nascent polypeptide. The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. (B) A progress of purification SDS-PAGE (10%) of recombinant ACTC proteins from insect cell culture using gelsolin segments 4-6 affinity chromatography. ACTC proteins in cell lysates are bound to His-tagged G4-6 (lane 1) before being applied to a Ni-NTA column. The flow through and resulting washes are shown (lanes 2-4). ACTC protein is released from the Ni-NTA resin in a buffer containing EGTA and bound to a DEAE column attached in series. The bound G4-6 protein is eluted from the Ni-NTA column in high imidazole (lanes 5-6), while the ACTC protein is eluted from the DEAE column with salt (lanes 7-9). Relative protein concentrations in elution fractions were determined using Bio Rad protein dye in samples and the highest concentration fractions were visualized on the gel. In both G4-6 and ACTC elutions, fraction 1 did not contain a high concentration of protein and was not run on the gel. See *Methods and materials* for details.

Figure 2: Biochemical analysis of ACTC variants. (A) Michaelis-Menten plots of recombinant ACTC proteins. Best fit curve parameters are presented in Table 1. WTrec, H88Y, F90Δ, and R95C ACTC plots represent data from 2 biological replicates with error bars representing the SEM. E99K ACTC data is from 1 biological replicate with error bars representing SEM. *inset*, Michaelis-Menten plots comparing WT ACTC protein from bovine cardiac tissue and WTrec

438 proteins. Tissue-purified ACTC protein data is from 1 biological replicate. Post hoc Dunnet's
 439 statistical testing was used to determine significant differences. (B) Box plots of *in vitro* motility
 440 velocities of ACTC variants. Kruskal-Wallis statistical analysis showed there were no significant
 441 differences between WTrec (N=9) to R95C ($p>0.05$, N= 11) and WTrec to H88Y ($p>0.05$, N=
 442 9). There were significant differences between WTrec to F90Δ ($*p<0.05$, N= 8), and WTrec to
 443 E99K ($*p< 0.05$, N= 5)

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Draft

Figure 1: (A) A ribbon model of actin (PDB 1ATN) visualized using PyMol showing the locations of HCM-related amino acid changes in the predicted myosin binding site. Bound ATP is shown as sticks. The amino acid numbers listed are based on the sequence of actin following posttranslational modification of the N-terminus removing the first two amino acids of the nascent polypeptide. The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC. (B) A progress of purification SDS-PAGE (10%) of recombinant ACTC proteins from insect cell culture using gelsolin segments 4-6 affinity chromatography. ACTC proteins in cell lysates are bound to His-tagged G4-6 (lane 1) before being applied to a Ni-NTA column. The flow through and resulting washes are shown (lanes 2-4). ACTC protein is released from the Ni-NTA resin in a buffer containing EGTA and bound to a DEAE column attached in series. The bound G4-6 protein is eluted from the Ni-NTA column in high imidazole (lanes 5-6), while the ACTC protein is eluted from the DEAE column with salt (lanes 7-9). See Methods and materials for details.

147x160mm (300 x 300 DPI)

Figure 2: Biochemical analysis of ACTC variants. (A) Michaelis-Menten plots of recombinant ACTC proteins. Best fit curve parameters are presented in Table 1. WTrec, H88Y, F90Δ, and R95C ACTC plots represent data from 2 biological replicates with error bars representing the SEM. E99K ACTC data is from 1 biological replicate with error bars representing SEM. inset, Michaelis-Menten plots comparing WT ACTC protein from bovine cardiac tissue and WTrec proteins. Tissue-purified ACTC protein data is from 1 biological replicate. Post hoc Dunnet's statistical testing was used to determine significant differences. (B) Box plots of in vitro motility velocities of ACTC variants. Kruskal-Wallis statistical analysis showed there were no significant differences between WTrec (N=9) to R95C ($p > 0.05$, N = 11) and WTrec to H88Y ($p > 0.05$, N = 9). There were significant differences between WTrec to F90Δ (* $p < 0.05$, N = 8), and WTrec to E99K (* $p < 0.05$, N = 5).

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