SUPPLEMENTARY METHODS AND FIGURES

Titin Based Viscosity in Ventricular Physiology: An Integrative Investigation of PEVK-Actin Interactions

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DETAILED SUPPLEMENTAL METHODS

SKINNED CELL PREPARATION AND MECHANICS

Cardiomyocytes were isolated from 6 WT and 6 PEVK KO mice as previously described[1]. Briefly, mice were injected with 100 units heparin IP 10 minutes prior to isolation. Mice were anesthetized via isoflurane inhalation, sacrificed via cervical dislociation, and hearts were rapidly removed and cannulated. Langendorff perfusion was begun with a constant flow perfusion using a 37^{o}C calcium free HEPES buffered solution ([in mmol/L] 10 HEPES; 133.5 NaCl, 5 KCl, 1.2 NaH_2PO_4; 1.2 $MgSO_4$, 11 glucose, 4 Na-Pyruvate with 2.5U/L insulin, pH 7.44). After 5 minutes, this calcium free solution was replaced with a digestion solution (HEPES buffered solution with 297U/mg Type II Collagenase (Worthington Biochemical Company, Lakewood, NJ) and 25μmol/L $CaCl_2$ for activation of the collagenase). Hearts were allowed to digest for approximately 10 minutes then washed for 10 minutes with calcium free HEPES buffered solution with 20mmol/L BDM to prevent crossbridge cycling. Hearts were then removed and placed in a small dish containing calcium free HEPES solution with 20mmol/L 2,3-Butanedione monoxime (BDM) and high concentration protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF). The right ventricles and atria were removed, and myocytes were then mechanically dissociated from the LV. Cells were placed in a 15mL tube and allowed to pellet. The supernatant solution was removed and cells re-suspended in a relaxing solution ([in mmol/L]: 10 BES, 10 EGTA, 6.56 $MgCl_2$, 5.88 Na-ATP, 1 DTT, 46.35 potassium-propionate, 15 creatine phosphate) with 0.3% Triton X-100 (Thermo Scientific, Waltham MA) with a high concentration of protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF). Cells were incubated for 6 minutes then pelleted in a centrifuge at 4^{o}C for 3 minutes at 400RPM and again washed and re-suspended in relaxing solution buffer and stored on wet ice.

Myocytes were added in a room-temperature flow-through chamber with relaxing solution, mounted on the stage of an inverted microscope. One end of a single cell was glued to a force transducer (Model 406A or 403A, Aurora Scientific). The free end was then bent with a pulled glass pipette attached to micromanipulator so that the myocyte axis aligned with the microscope optical axis and the cross-sectional area (CSA) was measured. The free end of the cell was released and glued to a high-resolution motor (308B, Aurora Scientific, Aurora, Ontario, Canada). Sarcomere length was measured using a CCD Camera and IonWizard Software (Ionoptix, Cambridge MA). Cells were activated using a pCa 4.5 activating solution ([in mmol/L] 40 BES, 10 $CaCO_3$ EGTA, 6.29 $MgCl_2$, 6.12 Na-ATP, 1 DTT, 45.3 potassium-proprionate, 15 creatine phosphate) and protease inhibitors ([in mmol/L] 0.01 E64, 0.04 Leupeptin and 0.5 PMSF) to confirm cell viability and allowed to rest for 15 minutes. All mechanical protocols were performed in relaxing buffer and protease inhibitors at room temperature (24^{o}C).

To ascertain the speed dependence of cardiomyocyte viscosity, myocytes were stretched from slack length to 2.15 μm, held for 20 seconds, and then released with ramp speeds of 0.01, 0.1, 1, and 10 lengths/s (Fig.1). All stretch protocols were carried out under sarcomere length control using constant strain rates. At least 7 minutes of recovery time in between stretches was utilized to prevent memory-effects in subsequent measurements. To further quantify viscosity, sinusoidal oscillations were imposed at a SL of 2.15 μm using a frequency sweep from 0.1Hz to 100Hz with an amplitude of +/-5% of the cell length. Data was collected using a custom LabVIEW VI (National Instruments, Austin TX) at a sample rate of 1kHz and stored offline.

MOUSE SKINNED MUSCLE FIBER PREPARATION AND MECHANICS

Skinned muscle fibers were obtained from WT, PEVK KO and N2B KO mice as previously described[1-3]. Briefly, hearts were quickly excised from heparinized, anesthetized mice, and

the LV opened along the septum and pinned to a Sylgard (Dow Corning, Midland MI)-coated Petri dish in oxygenated HEPES buffer. Papillary muscles were dissected and placed in a relaxing solution with 1% Triton X-100 and protease inhibitors ([in mmol/L] 0.1 E64, 0.4 Leupeptin and 0.5 PMSF) for 24-48 hrs at 4^{o}C with at least one solution exchange. The tissues were washed with Triton-free relaxing buffer for a minimum of 4 hrs, infiltrated with a 50% (vol/vol) solution of relaxing solution with protease inhibitors and glycerol for a minimum of 8 hrs (both at 4^{o}C), and then stored in this solution at -20^{o}C. Small fibers (~0.03mm^2 CSA and ~1.0 mm length) were dissected from the papillary muscles that were glued at their ends to aluminum clips. Fibers were attached to a force transducer (AE801, SensorOne, Sausilito CA) and length motor (308B Aurora Scientific). Cross sectional area (CSA) was calculated using the dimensions of two perpendicular measurements assuming an ellipsoid shape. To test fiber quality, all preparations were activated at sarcomere length 2.0 μm using a pCa 4.5 solution and achieved tensions of 43±2 (WT) and 39±3 (KO) mN/mm^2. Fibers were washed with relaxing buffer, allowed to rest for 15 minutes below their slack length, and where then returned to their slack SL. All mechanical protocols were performed in relaxing buffer with protease inhibitors ([in mmol/L] 0.01 E64, 0.04 Leupeptin and 0.5 PMSF) at room temperature. Cardiac muscle fibers were stretched using a stretch-hold-release protocol from their slack sarcomere length to a SL of 2.15 μm at 4 speeds: 0.1, 1.0, 10.0, and 50.0 lengths/s with at least 10 minutes of recovery time in between stretches. The fibers were held at their stretched length for 90 seconds, followed by a release. A sinusoidal frequency sweep was applied at the end of the 90 second hold of a 0.1 length/s stretch with frequencies from 0.1 to 400Hz with an amplitude of +/-2% of the fiber length. All stretch protocols were induced under sarcomere length control using constant strain rates. Myofilaments were extracted using a high KCl concentration (0.6 mol/L) relaxing buffer to depolymerize the thick filaments and high KI concentration (1.0 mol/L) buffer to depolymerize the thin filaments. Protocols were then repeated to determine ECM based stiffness. Data was collected from 6 animals per group and stored using a custom LabVIEW VI at a sample rate of 10 kHz and stored offline.

Because reports indicate that increasing temperature PEVK-actin binding[4], we performed the stretch-hold-release protocols at room temperature and at a physiological temperature of 37^{o}C. To examine the possibility of acto-myosin interactions affecting the viscosity results, preparations were also examined in the absence and presence of blebbistatin, a molecule that uncouples myofilaments from calcium activation[5]. Stretch-hold-release protocols were repeated for all preparations as described above at both room temperature and 37^{o}C in relaxing buffer with protease inhibitors and in the presence and absence 10 μmol/L blebbistatin, a concentration that was confirmed to produce no measurable active force in pCa 4.5 solution[5]. Data was collected from 5 WT and 5 PEVK KO mice and compared to data acquired at room temperature without blebbistatin. The relaxing buffers were adjusted to pH 7.0 for each temperature.

Furthermore, tests to understand the changes in viscosity due to experimental conditions were performed. Because lattice spacing is known to expand upon skinning[6-7], we examined the effect of lattice spacing on viscosity by performing experiments in the presence of 3% dextran to compress the lattice close to the intact state[8]. We also examined potential additive effects of compressed lattice with physiological temperatures by performing stretch-hold-release protocols at 37^{o}C in the presence of 3% dextran T-500. To eliminate the possibility of residual acto-myosin interaction contributing to viscosity, experiments with dextran were performed in relaxing buffer with protease inhibitors inhibitors and 10 μmol/L blebbistatin, and followed by myofilament extraction at room temperature. Data was collected from 6 WT and 6 PEVK KO hearts compared to data acquired at room temperature without dextran. The relaxing buffers were adjusted to pH 7.0 for each temperature.

While diastolic calcium levels in-vivo are thought to be ~pCa 6.8, the relaxing solution has a low calcium concentration (~pCa 9.0)[9-12]. To test the influence of calcium, WT and PEVK KO fibers were first mechanically stretched with the relaxing solution, then incubated in a pCa 6.8 and protease solution for 20 minutes. The mechanical experiments were repeated and compared to baseline.

ANALYSIS OF SKINNED MYOCYTE AND MUSCLE MECHANICS

Data was analyzed off line in a custom LabVIEW VI. Stress was calculated by dividing measured force by cross sectional area. For stretch-hold experiments, stress data was taken at the end of the stretch to calculate the peak stress, and again at the end of the hold (20 s after peak for cells; 90 s after peak for muscle fibers) to calculate a steady state stress. Analysis methodology was adapted from previous work myocardial tissues[13-14]. The viscous stress is calculated as the peak minus steady state stress during the hold {see inset of Fig.1B}. The coefficient of viscosity has been shown to be non-Newtonian (nonlinear relation of viscosity vs. stretch speed)[14-15] and our data shows a logarithmic relationship. To simplify the calculation, a coefficient of viscosity was calculated as the slope between the viscous stress and the $\log_{10}$ of the stretch speed (in lengths/s).

Frequency sweep data was analyzed to obtain viscous moduli[16]. Briefly, the stress was used to first calculate the complex stiffness as the stress divided by the % length change. Phase delay was calculated as the phase difference between the stress and strain signals. (For myocyte mechanics, the internal timing/signal processing delay for the force transducer was determined as 12 ms using a glutaraldehyde fixed cell and measured phase was corrected for this delay; no delay was found for the fiber mechanics in the frequency range that was used.) The viscous modulus was then calculated as complex stiffness times the sine of the phase delay[16] (see inset of Fig.1C). To correct for prep length, strain was normalized to cell or fiber length (L').

ISOLATED HEART MECHANICS AND ANALYSIS

Isolated perfused mouse hearts were prepared as previously described[1, 3]. Briefly, 10 WT and 10 PEVK KO mice were heparinized and anesthetized. Mice were sacrificed (as above) and the hearts quickly cannulated for Langendorff perfusion. Constant pressure (90mmHg) perfusion was provided with an oxygenated 37^{o}C Tyrode solution ([in mmol/L] 21 $NaHCO_3$, 7.4 KCl, 127 NaCl, 1.08 $MgCl_2$, 0.36 NaH_2PO_4, 1.75 $CaCl_2$, 5 Glucose, 5 Na-Pyruvate, with 5 U/L Insulin and 80 mg/L Bovine Serum Albumin [BSA]). Left and right atria were opened for access and the AV-node was heat ablated. A small needle was used to guide a custom plastic balloon in the LV between the mitral valve orifice and LV apex. The heart was submerged in a temperature controlled chamber filled with Tyrode perfusate. The balloon was connected to a chamber containing degassed water attached to a servo motor for volume control, and a micromanometric pressure catheter (SPR-471, Millar Instruments, Houston TX). The balloon was inflated to a slightly preloaded volume achieving a diastolic pressure of 5-10 mmHg, and the ventricle was externally paced via field stimulation by platinum electrodes at 200 bpm, typically developing ~90 mmHg developed pressure. The heart was then allowed to equilibrate for approximately 5 minutes. For the ramp-hold perturbation, pacing was extended to a 450 ms period for a single beat creating an approximately 150 ms diastolic plateau. Ramps were produced at a time-point in the cardiac cycle after the pressure achieved a diastolic plateau {Fig. 4A} and provided a stretch-hold protocol with approximately 100ms of hold time. We utilized a 10 ms step stretch of 2.5% of the baseline (diastolic) volume, corresponding to approximately 1 length/s fiber stretch when assuming a spherical shape. This speed was chosen to balance the pacing limitations (0.1 length/s requires a ramp of ~100ms) and the limited step and frequency

response of the system. The frequency response of the system also limited oscillation frequencies to 60 Hz; furthermore, higher frequencies were avoided to prevent stretch activation/deactivation affects. We utilized oscillations at 10, 20, 30, 40, 50 and 60 Hz with an amplitude of 2.5% of the baseline volume. To provide for sufficient oscillation time, pacing period was extended to 500ms, providing approximately 200 ms of diastolic plateau to analyze. Volume control and data acquisition was performed using custom PC and stored for offline analysis.

To assess the importance of microtubules in intact hearts, we also perfused a subset of 6 WT and 4 PEVK KO hearts with 5 μM colchicine. Ramp-hold perturbations were initiated before perfusion and at 5, 10, 15, and 30 minute intervals during continuous colchicine perfusion. To determine tubulin expression, qRT-PCR was used. RNA from a separate cohort of ventricles (7WT/7KO) was isolated and amplified with TaqMan probes (Applied Biosystems) for Tubulin and 18S RNA (for normalization) as described previously[17]. To visualize the microtubule polymerization via immunoflourescence staining, cardiomyocytes from adult wild type and PEVK deficient animals were seeded to laminin-coated glass coverslips (4h) and fixed with 4% PFA. Primary antibody: monoclonal mouse anti alpha-Tubulin DM1A (Calbiochem), for visualization of F-Actin fibers was an Alexa Fluor 488 conjugated Phalloidin (Molecular Probes) used. Secondary antibody was Alexa Fluor Cy3 conjugated goat anti mouse (Molecular Probes). Stained cells were analyzed on a confocal scanning laser microscope (Leica, SP2) with a 63X lens.

All data from isolated hearts were analyzed in a custom LabVIEW interface. Pressures were corrected with heart-removed balloon-measurements at identical volumes. Pressures were converted to wall stress by utilizing the equation:

$$Stress = \frac{P}{\left(1 + \frac{W_{LV}/1.05}{V_{LV}}\right)^{2/3} - 1}$$

where W_{LV} is the ventricular weight and V_{LV} is the ventricular volume[1]. The viscous stress was determined from stretch hold experiments as peak minus steady state wall stress as described above. Volume oscillations were also analyzed for complex stiffness and viscous modulus as described above, replacing volume for length/strain.

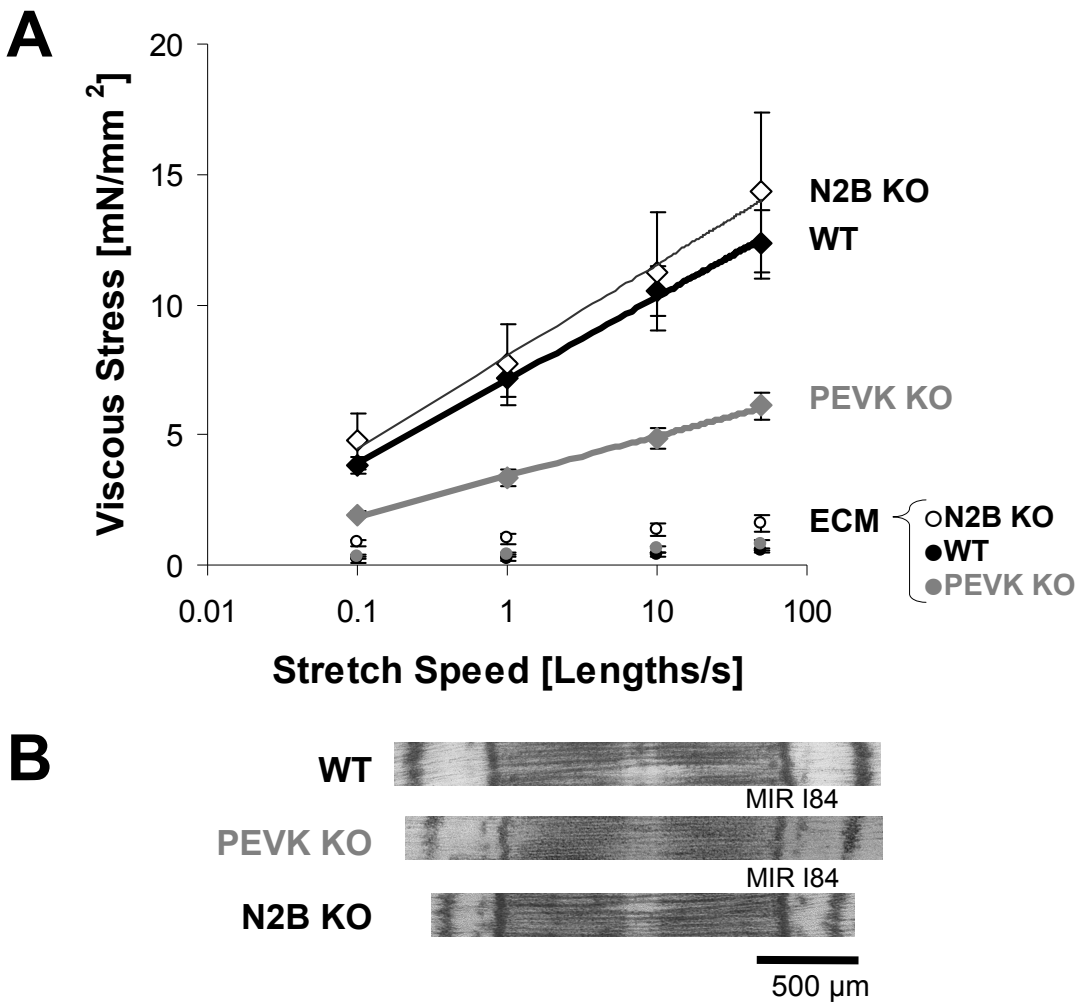
IN-VIVO ECHOCARDIOGRAPHIC EXPERIMENTS

Doppler echocardiography provides a method to examine the effects of viscosity on in-vivo cardiac function. Echocardiographic data was acquired using a Vevo 770 small animal echocardiography imaging system (VisualSonics, Toronto, Ontario). 7 WT and 7 PEVK KO were anesthetized under a 1% isoflurane/oxygen mixture. Left ventricular inflow Doppler alignment was obtained from the subcostal position. Analysis focused on Doppler inflow during diastolic early rapid filling and was done on a custom LabVIEW interface. Deceleration time (DT) measurements were taken according to American Society of Echocardiography Guidelines[18-19]. Preload (mean Left Atrial pressure, Left Atrial size, or blood pressure) was not directly measured in these mice. Acceleration time (AT) measurements were also taken[18, 20]. Shmulovich and Kovács[21] recently found that DT is not an independent measure of stiffness as previously described[22]. In fact, a decrease in DT could be caused by either an increase in stiffness or a decrease in viscosity. Similarly: a decrease in AT can be caused by an increase in stiffness or increase in viscosity. Only when DT-AT=0 is DT directly related to stiffness because viscosity is eliminated, suggesting that increasing DT-AT is an index of viscosity. Because both AT and DT can be modulated by changes in both stiffness and

viscosity, we also made quantitative, independent viscosity measurements according to the Parameterized Diastolic Filling Formalism[23]. This formalism provides viscosity and stiffness parameters via a kinematic model that are more sensitive than traditional DT estimates[21, 24]. Briefly, the E-wave velocity vs. time is taken and entered into a custom LabVIEW interface. Parameters are estimated via a Levenberg-Marquardt Algorithm providing mass-normalized stiffness (k) and viscosity (c) parameters (see[21, 23-24] for details). These parameters provide quantitative methods for examining in-vivo physiology.

STATISTICS

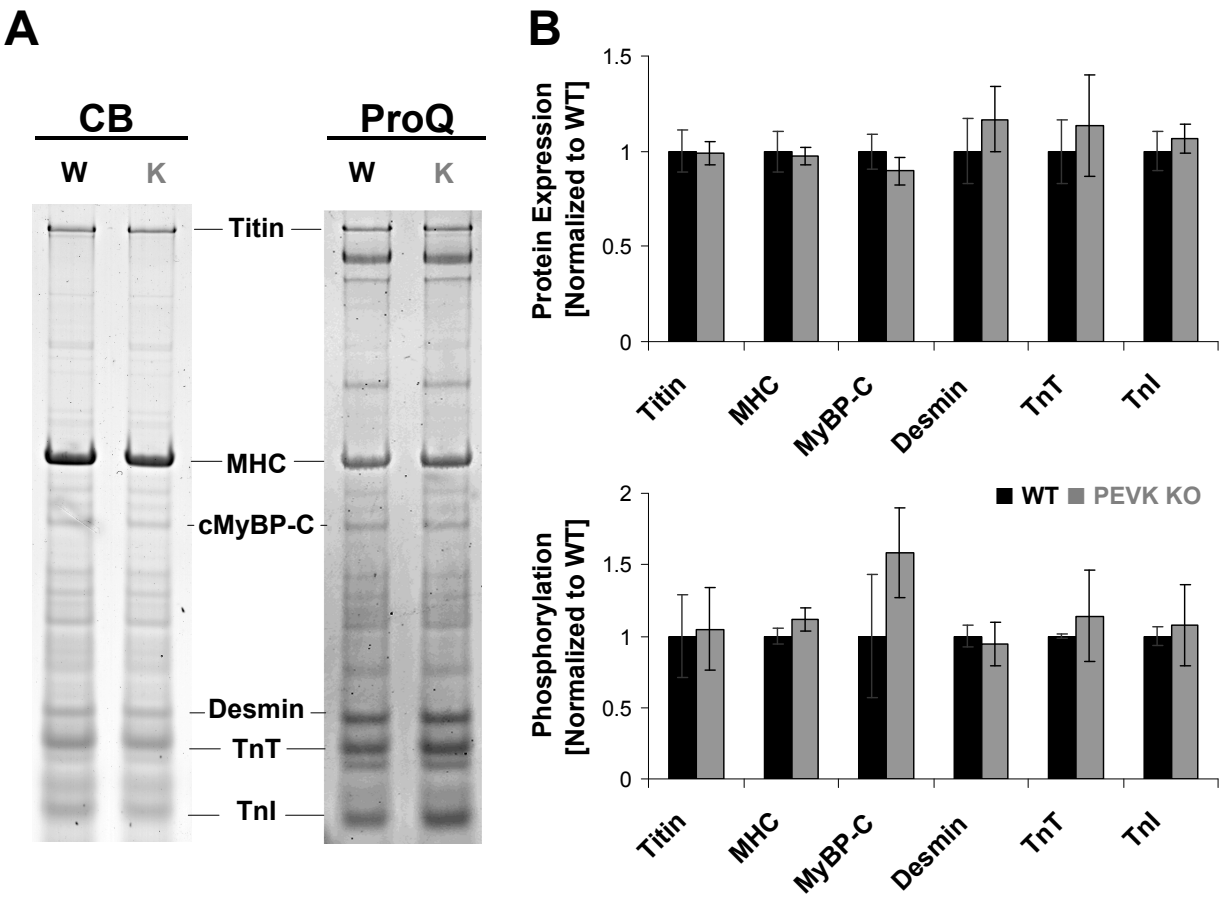
Additional data analysis and statistics were compiled in MS EXCEL 2003 or GraphPad Prism 5. Comparisons between two groups were with Student t-tests. ANOVA was used for comparisons between multiple groups with the following post-hoc tests: Kruskal-Wallis test with Dunn's post test was used when data was non-parametric (Supplemental Fig.1C) while parametric data was analyzed using ANOVA (with repeated measures where applicable) and Bonferroni post-hoc test.



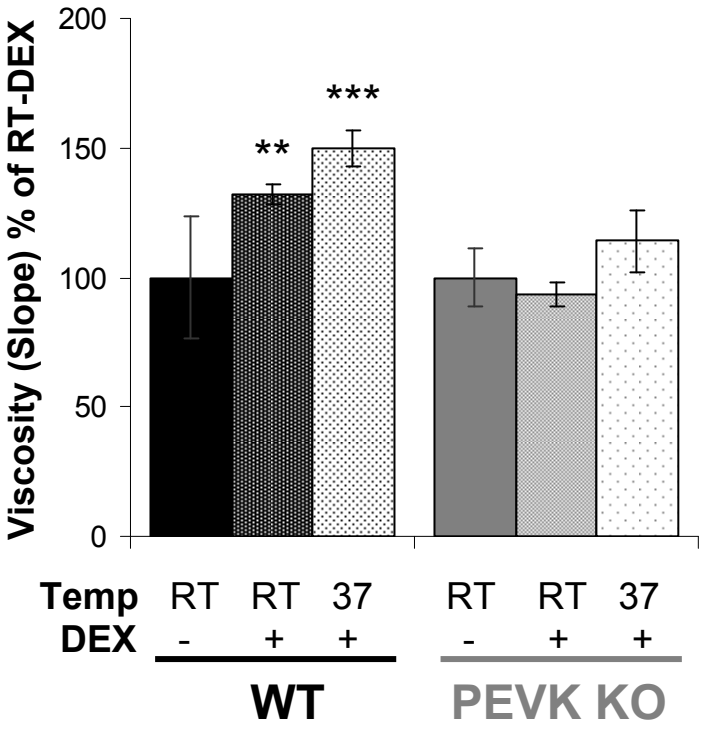
C

	Viscosity (Slope)	Maximal Active Tension [mN/mm^2]
WT	3.19 ± 0.38	43 ± 2
PEVK KO	1.54 ± 0.19*	39 ± 3
N2B KO	3.17 ± 0.65†	48 ± 4

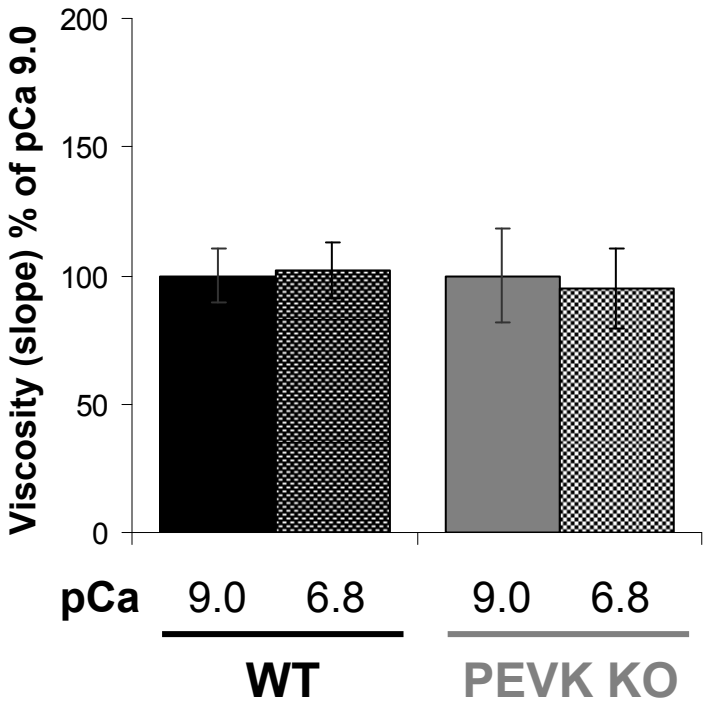
Supplemental Figure S1. Viscosity in N2B KO muscle. A) Comparison of passive viscous stress in N2B KO (open diamonds), WT (closed diamonds), PEVK KO (gray diamonds), papillary muscle (WT and PEVK KO same as Fig.2C). Viscosity of N2B KO is similar to that of WT, whereas viscosity in the PEVK KO is reduced. Collagen does not significantly contribute to viscosity in any of the three genotypes. B) Immunoelectron microscopy comparison of WT, PEVK KO and N2B KO sarcomeres labeled for MIR and I84 epitopes. No obvious ultrastructural differences are apparent. C) Table comparing slopes (viscosity) and maximum active tension between WT, PEVK KO and N2B KO fibers. *p<0.05 vs WT; † p<0.05 vs PEVK.



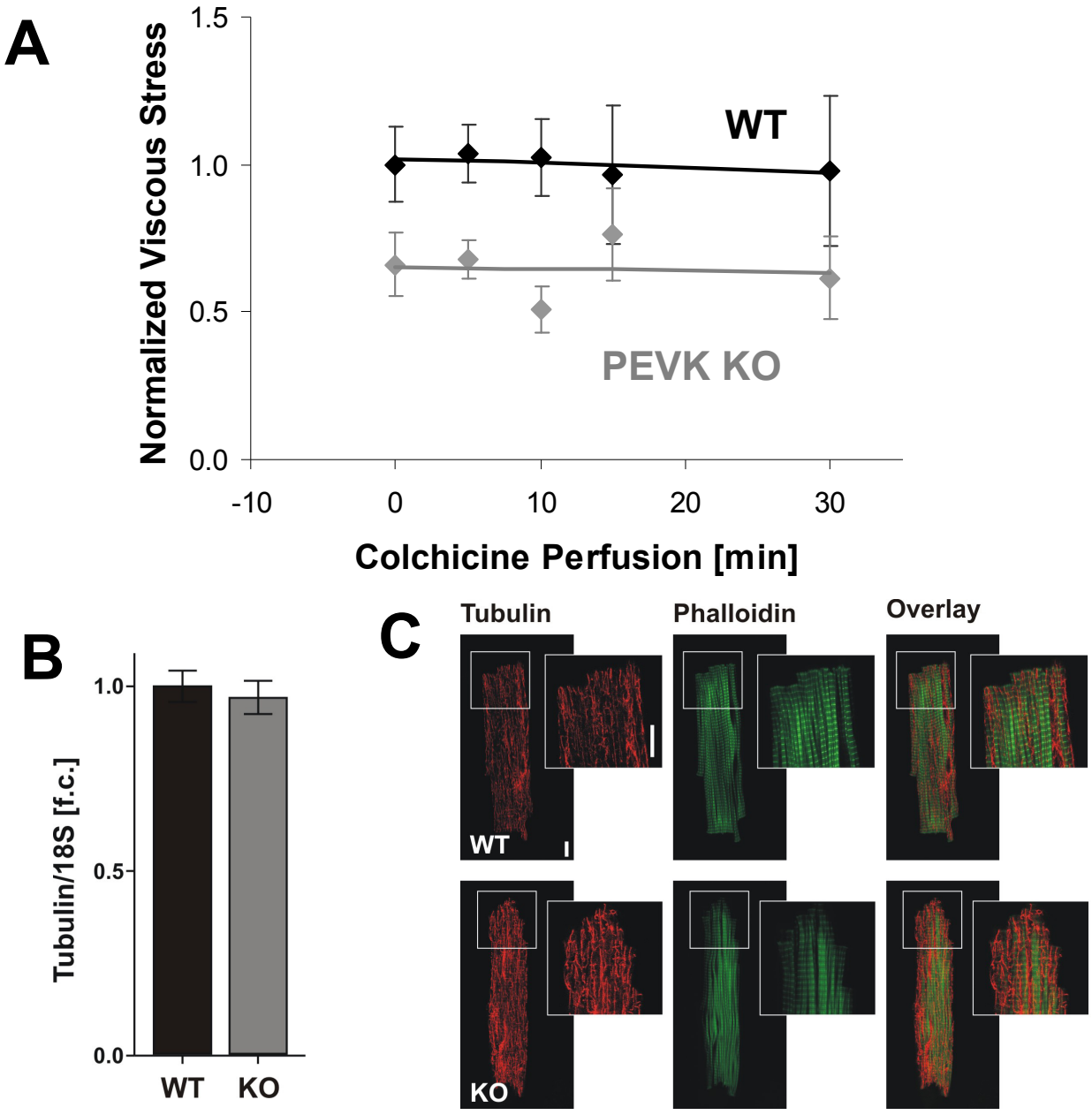
Supplemental Figure S2. Protein expression and phosphorylation in the PEVK KO. A) 2-7% SDS-PAGE and ProQ Diamond staining in WT and PEVK KO tissues. B) Quantification of protein expression and phosphorylation normalized to WT; no differences between WT and PEVK KO hearts were found.



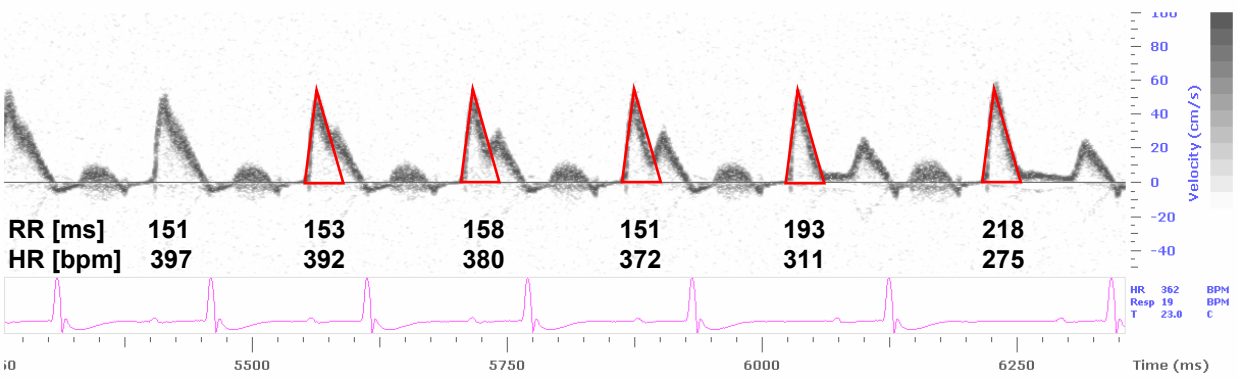
Supplemental Figure S3. Effect of 3% Dextran and temperature on the passive coefficient of viscosity in skinned cardiac tissue in the presences of blebbistatin. Compare to control (RT, -), the addition of dextran (RT, +) caused an increase in coefficient of viscosity in WT ($p<0.01$), but not KO myocardium. Adding dextran and increasing temperature (37 °C, +) increased the coefficient of viscosity in WT ($p<0.001$ vs RT, -), but there was no significant difference from dextran at room temperature. No effects were seen in KO tissues.



Supplemental Figure S4. Calcium effect on viscosity (at room temperature). Increasing calcium concentration from pCa 9.0 to 6.8 causes no change in viscosity in either PEVK WT or KO papillary muscles.



Supplemental Figure S5. Effect of colchicine on viscosity. A) Treatment by colchicine in isolated mouse hearts showed no difference in viscous stress. B) Tubulin mRNA expression (normalized to 18S) shows no difference between WT or PEVK KO hearts. C) The structure of the tubulin network was similar in cardiomyocytes from PEVK KO and WT animals. Tubulin was stained with an antibody against alpha-tubulin (red) and F-Actin was visualized with fluorescent labeled phalloidin (green). Size bar 10μm.



Supplemental Figure S6. Effect of heart rate on E-wave. Continuous image acquisition in a mouse with consecutive beats at varying heart rates. All red triangles have a DT (E-wave deceleration time) of 27ms and an AT (E-wave acceleration time) of 12ms and are identically overlaid on all beats. This indicates that the E-wave is not heart rate dependent. Note that at high HR (left most triangle) the E-wave becomes essentially indistinguishable from the A-wave due to superposition and it becomes difficult to measure the DT. Image color/contrast adjusted for clarity and ECG-derived R-R intervals and HR are reported.

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