SUPPLEMENTAL MATERIAL

MOLECULAR MECHANISM OF THE Glu99Lys MUTATION IN CARDIAC ACTIN (*ACTC* GENE) THAT CAUSES APICAL HYPERTROPHY IN MAN AND MOUSE

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SUPLEMENTAL METHODS

Generation of transgenic mice

Cardiac actin cDNA was provided by Dr. Kristen Nowak (University of Western Australia) in a pBSII KS vector. The E99K mutation was inserted into the human cardiac actin sequence by Dr. Charles Redwood (University of Oxford). Transgene expression was restricted to the heart using an alpha-myosin heavy chain promoter construct kindly provided by Dr. Mike Gollob and originally produced in the laboratory of Dr. Jeffrey Robbins (Subramaniam *et al*., 1991). In this construct, the promoter region of alpha-myosin heavy chain starts with the 3' untranslated region of the beta-myosin heavy chain and the first three exons of alpha-myosin heavy chain that are non-coding. The transgene expression vector was first cut with Hind III then partially filled in with two nucleotides (AG) using Klenow fragment. After purification the vector was then digested with Sal1. The mutant cardiac actin cDNA was removed from the pBSII KS vector by first digesting with Spe I, then filling the 3' end with two nucleotides (C,T) using Klenow fragment. After purification the vector was digested with Sal1. This allowed directional cloning of the mutant actin construct into the transgene expression vector using a Sal1 site at the 5' end and a Spe1/Hind III hybrid at the 3' end. Transgenic mice were generated by pronuclear microinjection of gel purified transgenic constructs (after removal of the plasmid backbone with Not I) into the pronucleus of fertilised mouse eggs on a C57BL10xCBA/Ca hybrid background as previously described (1,2) Treated embryos were returned to a pseudopregnant CD-1 foster mother, generated by mating with a vasectomised male mouse, and the resultant pups were identified and genotyped by PCR from ear notch samples.[7]

Characterisation of mutant actin

Mutant actin in transgenic mouse heart was identified and quantified by 2-dimensional electrophoresis using the method described by Ilkovski *et al.* (3). 15μg of mouse heart (wet weight) was solubilised and separated by isoelectric focusing in the first dimension (18cm pI 4-7 Immobiline DryStrip gels; GE Biosciences) and SDS-PAGE in the second dimension. Actin was detected in western blots probed with 5C5 anti-sarcomeric actin monoclonal antibody (Sigma) and visualized by Enhanced Chemiluminescence (ECL; GE Biosciences).

Echocardiology

Mice were secured on the temperature regulated plate (to maintain body core temperature at 37±0.5°C, measured

with rectal temperature probe) at a supine position with 1 to 1.5% isoflurane anaesthesia. Limbs were taped to copper leads for electrocardiogram gating. The animals were imaged with a 30MHz linear probe (VisualSonics Vevo 770 with a RMV 707B scanhead). Two-dimensional images were recorded in parasternal long- and short-axis projections with guided M-mode recordings at the midventricular level in both views. Left ventricular (LV) cavity size and wall thickness were measured in at least three beats from each projection and averaged. LV wall thickness [interventricular septum (IVS) and posterior wall (PW) thickness] and internal dimensions at diastole and systole (LVIDd and LVIDs, respectively) were measured. LV fractional shortening [(LVIDd – LVIDs)/LVIDd] is calculated from the M-mode measurements. Dobutamine (1.5 mg/kg) was injected i.p. and images were recorded at 5 minutes before and 5 minutes after the injection.

ECG

Mice were secured on the temperature regulated plate (to maintain body core temperature at 37 ± 0.5°C measured with rectal temperature probe) at a supine position with 1 to 1.5% isoflurane anaesthesia. Lead II ECG was recorded and analysed with PowerLab and LabChart 7.0 Pro (ADInstruments). The subclavian vein was used to infuse 2.5mg/kg bw isoprenaline. Data were collected 5 minutes before and 10 minutes after bolus isoprenaline infusion.

Magnetic resonance imaging

In vivo MRI was performed as described (4). Animals were anaesthetised with 1.5 - 2.5% isoflurane in O_2 and positioned supine in a purpose-built, temperature-regulated cradle. ECG electrodes were inserted into the forepaws and a loop of wire was taped across the chest to monitor respiration. The cradle was lowered into a vertical bore 500 MHz, 11.7 T MR magnet with a Bruker console and a 40 mm birdcage RF coil (Rapid Biomedical, Würzburg, Germany). Long and short-axis scout images were acquired so that true short axis images could be planned using a segmented, ECG-triggered FLASH-sequence. The RF coil was then tuned and matched, followed by slice-selective shimming. Cine-MR images, consisting of 20 to 30 frames per heart cycle, were acquired in seven to eight contiguous slices in the short-axis orientation covering the entire heart. The imaging parameters were, field of view 25.6 × 25.6 mm, matrix size 256 × 256, slice thickness 1 mm giving a voxel size 0.0025 mm^3, echo time/repetition time = 1.43/4.6 ms, 0.5 ms/17.5° Gaussian RF excitation pulse, 4 averages. The total experimental time, including animal preparation, was approximately 50 minutes per animal. End-diastolic and end-systolic frames were selected as those with largest and smallest cavity volumes, respectively. Epicardial and endocardial borders were outlined using the free-hand drawing function of Scion Image (Scion Corporation, Frederick, Maryland, USA). Measurements from all slices were summed to calculate end diastolic volume (EDV), end systolic volume (ESV), stroke volume (SV = EDV – ESV), ejection fraction (EF = SV/EDV), cardiac output (CO = SV × heart rate) and cardiac index (CI = CO/body mass). LV mass was calculated as myocardial area × slice thickness × myocardial specific gravity (1.05).

Conductance catheter

in vivo cardiac function was assessed in anesthetized mice by pressure and volume measurements, using a Mikro-Tip® pressure-volume catheter (Millar Instruments, TX, USA) and recoded with P-V conductance system (Millar Instruments) coupled to a PowerLab (AD Instruments, CA, USA) and a personal computer. The mouse was placed

supine on a thermoregulated surgical table at $37°C \pm 0.5°C$. Ventilation via endotracheal tube was maintained with 100% oxygen using a small animal ventilator delivering a tidal volume of 7 µl/g at 200 breaths /min. Anaesthesia was induced with 5% isoflurane. The subclavian vein was exposed for saline injection. The apex of the heart was exposed via an anterior thoracotomy with 2.5% isoflurane. With a 30g needle, a small hole was made in the apex to insert PV catheter (1.0F) and the catheter were secured with suture on the skin after being advanced into the right position. Anaesthesia was then maintained with 1-1.5% isofurane. Calibration of the parallel conductance was performed using injection of 10% hypertonic saline via subclavian vein.

PV loop analysis was made with analysis program PVAN 4.0 (Millar Instruments). Heart rate, maximal LV systolic pressure (ESP), LV end-diastolic pressure (EDP), maximal first derivative of systolic pressure with respect to time (dP/dt_{max}) and the peak negative rate of change of diastolic pressure (dP/dt_{min}), time constant of LV pressure decay (τ), ejection fraction (EF), stroke volume (SV), end-diastolic volume (EDV), cardiac output (CO), and stroke work (SW) were computed. Left ventricular pressure-volume relations were also assessed by transiently compressing the inferior vena cava. Indices of contractility, for example, preload recruitable stroke work (PRSW), and slope of end-systolic P-V relationship (ESPVR) were calculated.

Ca^{2+}-sensitivity of skinned mouse papillary muscle

Papillary muscles were dissected from the left ventricle of fresh hearts. Dissection was performed in oxygenated Krebs-Henseleit solution and 30mM 2,3-butanedione monoxime (BDM). T-shaped aluminium foil clips were gently attached to the ends of the isolated papillary muscles to allow them to be mounted on the experimental apparatus. The papillary muscles were then chemically skinned in a relaxing solution (100mM TES, 7.7mM $MgCl_2$, 25mM EGTA, 19.11mM Na_2CP, 5.44mM Na_2ATP, 10mM Glutathione) containing 2% Triton X-100 for 30 minutes. After permeabilisation, muscles were either used immediately or stored in a relaxing solution containing 50% glycerol at -20°C for up to 5 days (4).

The muscle was mounted, in relaxing solution at 20°C, between a force transducer and a motor on a rapid solution exchange system driven by a stepper motor. Laser diffraction was used to set resting sarcomere length to 2.1µm. The solution exchange system consists of 2 movable plates with pedestals to support the drops of solution, allowing quick transfer of the muscle between different solutions.

Contraction was initiated by a temperature-jump protocol.(5). One plate is used to transfer the muscle between preactivating solution (100mM TES, 6.93mM $MgCl_2$, 0.1mM EGTA, 24.9mM HDTA, 19.49mM Na_2CP, 5.45mM Na_2ATP, 10mM Glutathione) and activating solution (100mM TES, 6.76mM $MgCl_2$, 25mM CaEGTA, 19.49mM Na_2CP, 5.49mM Na_2ATP, 10mM Glutathione) at ~0°C. The second plate was used to transfer the muscle between activating solution and relaxing solution at 20°C, with the transition between the two plates producing a rapid change of temperature. Most of the force develops after the temperature jump and rapid transfer back to relaxing solution allows multiple contractions to be performed on the same muscle without a reduction in maximal force production. All solutions were made up to 200 mM ionic strength and pH 7.1 at 20°C.

Isometric force was measured at various calcium concentrations (ranging from pCa 4.5 to pCa 7) at 20°C. After maximal activation 9-10 measurements were carried out at submaximal [Ca^{2+}] followed by a maximal activation. Submaximal force measurements were normalised to the maximal force values. Data were fit with the Hill equation:

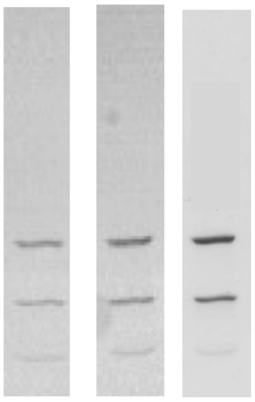
$P/P_0 = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)$ where P = steady-state force produced at each pCa, P_0 = maximal force at pCa 4.5, n = Hill coefficient (slope of force-pCa relationship) and k = EC_{50} ($[Ca^{2+}]$ at which 50% maximum force is produced).

Isolation of mouse polymeric actin

Polymeric actin was isolated from mouse hearts by a modification of the method of Tobacman and Sawyer (6,7). One heart (50-100mg) was pulverized in a liquid nitrogen cooled percussion mortar and homogenized in 10 x volume of wash buffer containing 20mM Pi, 0.1M NaCl, 5mM $MgCl_2$, 0.5 mM EGTA, 5mM DTT with 2 μ g/ml each of the protease inhibitors E-64, chymostatin, leupeptin and pepstatin A, pH 7.0. Samples were spun at 16,662xg for 3 minutes at 4°C. This wash step was repeated 4 times and the pellet was then extracted twice in 1.5 x volume of thin filament extraction buffer (wash buffer plus 5mM MgATP, 50μM blebbistatin (Sigma)) and centrifuged at 16,662xg for 3 minutes at 4°C. The two supernatants were combined and centrifuged at 111,700xg for 5 minutes at 4°C. The supernatant was further spun at 446,800xg for 20 minutes. The pellet containing thin filaments was resuspended and dialysed in 1.5 x volume of a buffer containing 20mM Pi, 0.1M NaCl, 5mM $MgCl_2$, and 1mM ATP, pH 6.0 for 3 hours at 4°C. A 2-minute spin at 16,662xg removed the remaining myosin. KCl was added to the supernatant to a final concentration of 0.8M to dissociate actin from troponin-tropomyosin. 150nM TRITC-phalloidin was also added to the supernatant. The dissociated thin filaments were spun at 337,000xg, for 20 minutes and the pellet containing the pure F-actin was resuspended in 100μl ACEX (2mM Tris-HCl, 0.2mM $CaCl_2$, 0.2mM ATP, 1mM DTT, pH8.0) overnight at 4°C.

Human cardiac troponin and tropomyosin

Human cardiac troponin was prepared from donor heart myofibrils using an antibody affinity column as described by Messer *et al* (8). Typical troponin preparations are shown. Phosphorylation of troponin I and troponin T was measured by staining SDS-PAGE gels of troponin or myofibrils with Pro-Q Diamond phosphoprotein specific stain followed by Coomassie blue total protein stain as described (8) or by Phoshate affinity SDS-PAGE as described by Messer *et al* (9). Troponin was dephosphorylated by treatment with acid phosphatase (Sigma) as previously (8). Human cardiac tropomyosin was isolated as described by Knott *et al* (10).



In vitro motility assay

The *in vitro* motility assay technique was used to study TRITC-Phalloidin labelled actin (actin-ϕ) filaments moving over immobilised rabbit fast muscle heavy meromyosin (4). The actomyosin system was reconstituted in a flow cell, constructed from a microscope slide and a siliconised coverslip. Actin-ϕ was pre-mixed with tropomyosin and troponin at 10× working concentration prior to dilution and infusion into the assay flow cell. Thin filament movement over a bed of immobilised rabbit fast skeletal muscle heavy meromyosin (100 μ g/ml) was observed in motility buffer D (50 mM KCl, 25 mM imidazole–HCl pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 0.5 mg/ml BSA, 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose, 0.5% methylcellulose, 5 mM Ca/EGTA buffer (range 3.7 μ M to 1 nM Ca^{2+}), 1 mM MgATP and troponin at the appropriate concentration. Filament movement was recorded and

analysed as previously described (11).

In vitro motility measurements were made in paired cells within two days of preparation of troponin. In the Ca^{2+}-concentration dependency measurements (Figs. 5 and 7, Table 2) the data were fitted to the 4-parameter Hill equation: $y = a + X_{max} [Ca^{2+}]^n / (EC_{50} + [Ca^{2+}]^n)$. We calculated the mean and standard error of each motility parameter (sliding speed, EC_{50} etc.). For measurements of Ca^{2+}-concentration dependence of motility we found variability between the absolute values of EC_{50} obtained with different troponin and HMM preparations, however the ratio of the EC_{50} values in control and *ACTC* E99K samples measured in paired experiments was consistent. We used the single group t-test to determine whether the ratio was significantly different from 1.

SUPPLEMENTAL DATA A

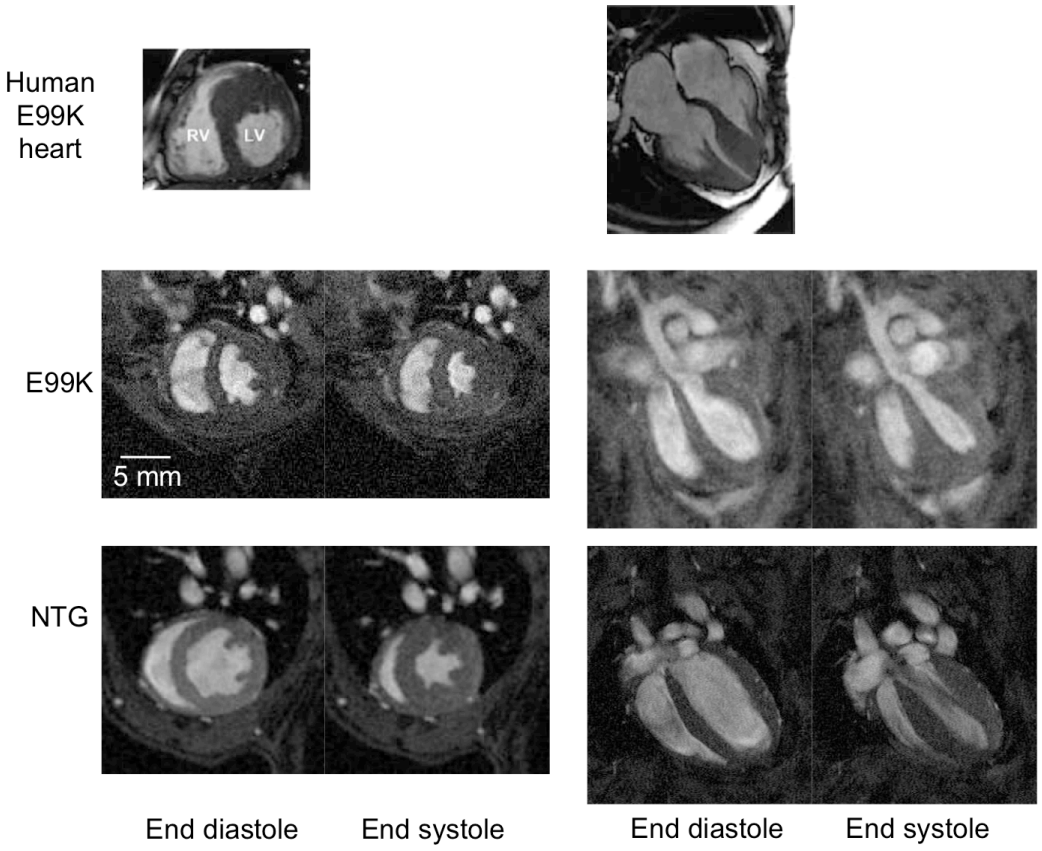
Clinical details of patients 1 and 2

Biopsy sample	**E99K 1**	**E99K 2**
Diagnosis	Hypertrophic cardiomyopathy-Non Compaction	Atrial septal defect
Age	55	33
Gender	F	M
Age when diagnosed	31	31
Age at operation	55	
operation	Heart transplant	Atrial septal defect closure
Family History	Multiple relatives with LVNC-Apical HCM	Multiple relatives with LVNC-Apical HCM, some with ASD.
Current Treatment	Betablockers: Bisoprolol Dose (mg/24h): Angiotensin coverting enzyme inhibitors (ACEI): Enalapril Dose (mg/24h): 5 Loop diuretics: · Furosemide Dose (mg/24h): 80 Potasium sparing diuretic: · EspironolactoneDose (mg/24h) Anticoagulation: Warfarine Digoxin: Dose (mg/24h): 0.2	aspirin
Max LVWT (mm)	17	26
Max. ST(mm)		
LVEDD (mm)	52	46
LVESD (mm)	32	29
LA (mm)	61	
FS (%)		37
Resting LVOT Gradient		
VT on Holter Monitor		
ECG	atrial fibrillation: Permanent Conduction system disease: Yes Pacemaker rhythm: Yes QRS complex Axis: Left Wideness: 82 QT interval QT duration (ms): 375 RR interval duration (s): 0.71 QT corrected interval in ms (according to Bazzet formula): 443.71	QRS complex Axis: Normal Wideness: 95 Abnormal voltage or repolarization: Yes Abnormal repolarization: Yes Low voltage: No LVH signs (left ventricle hypertrophy): No Left atrium Enlargement: No Right atrial Enlargement: No Negative T waves: No QT interval QT duration (ms): 368 RR interval duration (s): 0.8 QT corrected interval in ms (according to Bazzet formula): 411.44 Signs of right ventricular hypertrophy: No Q waves: No
NYHA Class	IV	

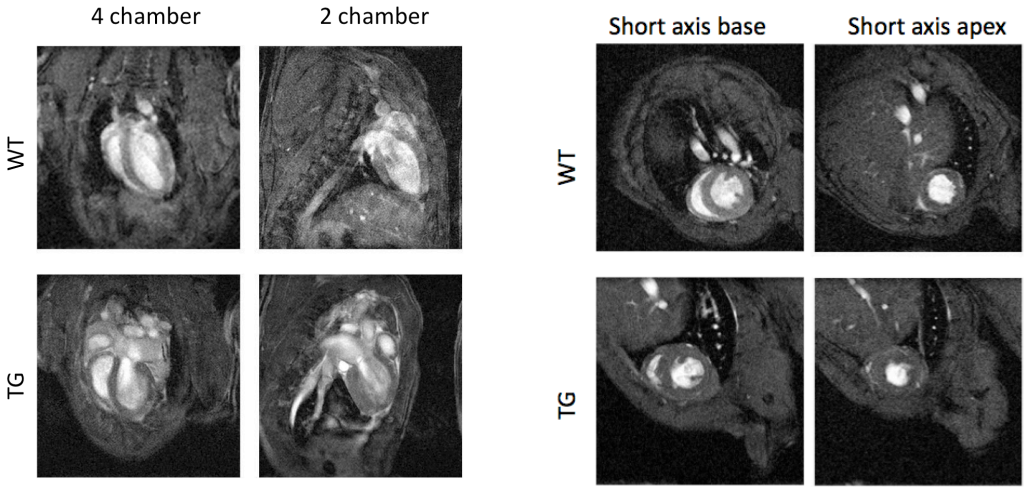
SUPPLEMENTAL DATA B

Imaging of mouse hearts by MRI

Bi 28 week-old male mice (E99K and non-transgenic littermates) and human heart comparison



Bii 21 week-old male mice



SUPPLEMENTAL DATA C

Ci Heart morphology and contractility measured by MRI

	21 weeks old male		29 weeks old male	
	NTG	ACTC E99K	NTG	ACTC E99K
No. of mice	8	7	7	9
HR (bpm)	427 ± 22	337 ± 8 ***	425 ± 15	400 ± 10
Body mass (g)	36 ± 1.77	35 ± 0.76	36.00 ± 1.13	36.11 ± 1
Heart mass:body mass, %	3.51 ± 0.29	3.77 ± 0.22	2.99 ± 0.14	2.74 ± 0.11
Left ventricular function				
EDV (µl)	66.04 ± 3.89	52.71 ± 3.40 **	65.15 ± 2.65	45.85 ± 2.33 ***
ESV (µl)	25.37 ±3.18	26.01 ± 1.51	22.85 ± 1.89	19.82 ± 1.33
SV (µl)	40.67 ± 2.83	26.69 ± 2.65 **	42.30 ± 1.51	26.04 ± 2 ***
EF (%)	62.19 ± 3.54	50.27 ± 2.27 **	65.16 ± 6.05	56.45 ± 2.33 **
CO (µl/min)	17.22 ± 1.06	9.06 ± 0.76 ***	18.12 ± 1.13	10.50 ± 1 ***
Right ventricular function				
EDV (µl)	51.52 ± 4.24	53.66 ± 3.02	59.29 ± 1.51	53.71 ± 3.67
ESV (µl)	11.00 ± 1.06	23.80 ± 3.02 ***	20.77 ± 2.27	27.95 ± 1.67 **
SV (µl)	40.51 ± 3.54	29.86 ± 1.13 **	38.52 ± 1.13	25.76 ± 2.33 ***
EF (%)	78.66 ± 1.41	56.32 ± 2.65 ***	65.29 ± 2.02	47.45 ± 1.67 ***
Wall thickness – base				
Septal thickness ED (mm)	1.03 ± 0.08	0.99 ± 0.06	1.03 ± 0.06	1,39 ± 0.05
Septal thickness ES (mm)	1.39 ± 0.06	1.38 ± 0.09	1.43 ± 0.07	1.39 ± 0.06
Free wall thickness ED (mm)	1.13 ± 0.09	1.21 ± 0.09	0.94 ± 0.06	1.17 ± 0.04 **
Free wall thickness ES (mm)	1.88 ± 0.10	1.74 ± 0.14	1.45 ± 0.09	1.56 ± 0.06
Septal thickening (mm)	0.36 ± 0.09	0.39 ± 0.06	0.40 ± 0.08	0.31 ± 0.04
Free wall thickening (mm)	0.76 ± 0.11	0.53 ± 0.09	0.51 ± 0.05	0.39 ± 0.07
Wall thickness - apex				
Septal thickness ED (mm)	1.06 ± 0.04	1.49 ± 0.12 ***	1.12 ± 0.07	1.51 ± 0.11 **
Septal thickness ES (mm)	1.62 ± 0.11	1.83 ± 0.04	1.62 ± 0.14	1.69 ± 0.09
Free wall thickness ED	1.11 ± 0.21	1.62 ± 0.23 *	0.96 ± 0.05	1.42 ± 0.07 ***
Free wall thickness ES (mm)	1.76 ± 0.13	1.77 ± 0.02	1.52 ± 0.11	1.68 ± 0.07
Septal thickening (mm)	0.58 ± 0.10	0.34 ± 0.06 *	0.50 ± 0.09	0.18 ± 0.06 **
Free wall thickening (mm)	0.64 ± 0.11	0.15 ± 0.12 **	0.56 ± 0.08	0.27 ± 0.05 **

Cii Heart morphology and contractility measured by Echo, 21 weeks male

Mid papillary Wall thickness, mm

	NTG	ACTC E99K
Septal thickness diastole	1.17 ± 0.06	1.14 ± 0.03
Septal thickness systole	1.55 ± 0.07	1.55 ± 0.04
Free wall thickness diastole	1.09 ± 0.08	1.49 ± 0.23
Free wall thickness systole	2.58 ± 0.19	2.56 ± 0.16

SUPPLEMENTAL DATA D

Conductance catheter measurements on 21 and 38 weeks old male mice

	21 weeks old male		38 weeks old male	
	NTG	ACTC E99K	NTG	ACTC E99K
No. of mice	5	6	4	5
HR (bpm)	558 ± 31	546 ± 22	537 ± 15	464 ± 26
EDV (µl)	21.31 ± 1.45	16.78 ± 1.30 *	22.82 ± 0.57	24.64 ± 1.228
ESV (µl)	7.212 ± 0.863	7.295 ± 0.407	7.723 ± 0.560	12.53 ± 1.31*
EDP (mmHg)	1.364 ± 0.610	5.693 ± 1.412 *	1.534 ± 0.184	2.787 ± 0.139 *
ESP (mmHg)	75.42 ± 3.43	73.89 ± 4.35	73.44 ± 5.88	69.08 ± 3.35
SV (µl)	14.10 ± 0.90	9.482 ± 1.178 *	15.10 ± 1.11	12.10 ± 1.45
EF (%)	66.37 ± 2.53	55.68 ± 2.74*	66.01 ± 3.31	49.00 ± 4.83 *
dP/dt_{max} (mmHg/sec)	8076 ± 871	8509 ± 741	6243 ± 853	5950 ± 356
dP/dt_{min} (mmHg/sec)	-7200 ± 957	-4710 ± 420 *	-6663 ± 788	-3046 ± 477
dV/dt_{max} (µl/sec)	787.6 ± 71.2	580.5 ± 98.0	888.0 ± 84.6	563.0 ± 50.2 *
dV/dt_{min} (µl/sec)	-810.6 ± 139.4	-531.8 ± 53.67	-848.0 ± 111.5	-555.4 ± 37.9 *
Tau_g (msec)	9.518 ± 1.023	17.72 ± 1.21 ***	13.32 ± 0.7167	30.89 ± 4.77 *
Ees (mmHg/µl)	12.08 ± 1.307	6.284 ± 1.231 *	-	-
PRSW ($mWatts/\mu l^2$)	80.00 ± 15.27	51.37 ± 3.99	-	-
EDPVR	0.217 ± 0.047	0.714 ± 0.116 **	-	-

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

SUPPLEMENTAL DATA E

Effects of dobutamine infusion, 21 weeks male mice, measured by conductance catheter

	Before dobutamine		After dobutamine	
	NTG	*ACTC* E99K	NTG	*ACTC* E99K
No. of mice	6	3	6	3
IVS;d (mm)	1.139 ± 0.034	1.172 ± 0.062	1.301 ± 0.072	1.184 ± 0.103
IVS;s (mm)	1.554 ± 0.044	1.544 ± 0.067	2.027 ± 0.053	1.550 ± 0.106 **
LVID;d (mm)	3.933 ± 0.161	3.468 ± 0.228	3.313 ± 0.119	3.523 ± 0.242
LVID;s(mm)	2.555 ± 0.164	2.579 ± 0.193	1.265 ± 0.116	2.512 ± 0.112 ***
LVPW;d (mm)	1.092 ± 0.081	1.486 ± 0.231	1.196 ± 0.0478	1.491 ± 0.224
LVPW;s (mm)	1.502 ± 0.099	1.607 ± 0.239	1.956 ± 0.078	1.599 ± 0.190
FS	35.19 ± 2.82	25.71 ± 1.45	61.92 ± 2.92	28.42 ± 2.45 ***

	% changes after dobutamine	
	NTG	ACTC E99K
No. of mice	6	3
IVS;d (mm)	13.03 ± 5.90	0.760 ± 4.960
IVS;s (mm)	28.41 ± 5.44	0.477 ± 6.516 *
LVID;d (mm)	-9.920 ± 7.351	1.573 ± 1.649
LVID;s (mm)	-50.48 ± 4.43	-2.072 ± 3.860 ***
LVPW;d (mm)	16.93 ± 12.03	1.89 ± 11.46
LVPW;s (mm)	36.74 ± 4.47	1.592 ± 9.919 **
FS	82.26 ± 18.38	11.23 ± 11.27 *

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

SUPPLEMENTAL DATA F

Conductance catheter measurements on 29 and 38 weeks old female mice

	29 weeks old female		38 weeks old female	
	NTG	ACTC E99K	NTG	ACTC E99K
No. of mice	4	4	4	5
HR (bpm)	423 ± 10	406 ± 20	588 ± 10	543 ± 12 *
EDV (µl)	22.31 ± 2.08	19.75 ± 0.29	21.20 ± 2.02	31.57 ± 1.86 **
ESV (µl)	7.488 ± 0.457	11.45 ± 0.36 ***	8.332 ± 0.8166	21.55 ± 0.55 ***
SV (µl)	14.83 ± 2.21	8.298 ± 0.306 *	12.87 ± 2.60	10.02 ± 2.01
EF (%)	65.44 ± 4.29	42.02 ± 1.50 **	58.92 ± 7.00	30.70 ± 4.71 **
EDP (mmHg)	1.364 ± 0.610	5.693 ± 1.412 *	1.406 ± 0.1694	2.091 ± 0.188 *
ESP (mmHg)	74.48 ± 5.81	71.03 ± 1.77	66.24 ± 5.00	65.21 ± 3.48
dP/dt_{max} (mmHg/sec)	6870 ± 554	5215 ± 829	6631 ± 488	6177 ± 1048
dP/dt_{min} (mmHg/sec)	-6338 ± 351	-4283 ± 459***	-6334 ± 689	-3078 ± 278 *
dV/dt_{max} (µl/sec)	787.6 ± 71.2	580.5 ± 98.0	660.8 ± 181.0	522.2 ± 58.5
dV/dt_{min} (µl/sec)	-810.6 ± 139.4	-531.8 ± 53.6	-703.8 ± 179.5	-536.0 ± 42.0
Tau_g (msec)	11.56 ± 0.80	33.67 ± 7.38*	10.51 ± 0.87	20.96 ± 1.89 **

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

SUPPLEMENTAL DATA G

Heart/body weight ratios, %

	21 weeks male	29 weeks male	29 weeks female	38 weeks male
ACTC E99K	3.77 ± 0.22	2.72 ± 0.34	5.16 ± 0.67	4.42 ± 0.26
NTG	3.51 ± 0.29	2.98 ± 0.35	3.68 ± 0.34*	3.35 ± 0.11*

* indicates P<0.05

SUPPLEMENTAL DATA H

Comparison of thin filaments containing E99K and NTG actin using motility assay

	number of hearts, paired	number of assays		Wild-type actin	E99K actin	P , paired t test
Actin	6	14	Sliding speed, µm/s	2.84±0.11	2.61±0.10	p=0.004
			Fraction motile	0.78±0.03	0.75±0.03	p=0.22
Actin.Tm	6	10	Sliding speed, µm/s	2.95±0.14	2.84±0.18	p=0.34
			Fraction motile	0.76±0.04	0.81±0.04	p=0.27
Actin.Tm.Tn (3.6µM Ca^{2+})	6	7	Sliding speed, µm/s	2.71±0.2	2.70±0.17	p=0.89
			Fraction motile	0.73±0.04	0.73±0.02	p=1.0
Actin.Tm.Tn (1 nM Ca^{2+})	6	7	Sliding speed, µm/s	1.86±0.10	1.81±0.11	p=0.65
			Fraction motile	0.13±0.04	0.11±0.03	p=0.53

SUPPLEMENTAL DATA I

Ca^{2+}-Sensitivity data for all Non-Transgenic and E99K Actin in a Non-Failing Thin Filament

Mouse		EC_{50} Percentage Motility, μM		$\frac{EC_{50}\ NTg}{EC_{50}\ E99K}$
Non-Transgenic	E99K	Non-Transgenic	E99K	
N2.3	N11.17	0.082 ± 0.002	0.035 ± 0.010	2.34
N11.30.9	N11.16	0.289 ± 0.114	0.074 ± 0.008	3.90
N2.16.30	N11.30.16	0.492 ± 0.162	0.292 ± 0.084	1.68
N5.3	N11.30.12	0.340 ± 0.064	0.170 ± 0.027	2.00
N5.9	N11.30.10	0.593 ± 0.127	0.212 ± 0.069	2.80
Mean: ± SE (n = 5)		0.359 ± 0.09	0.157 ± 0.05	2.54 ± 0.39
Student's t-test (paired)		p = 0.02		single group, p = 0.02 (compared with 1)
(Unpaired)		p = 0.07		

Ca^{2+} Sensitivity Data for E99K Actin Incorporated with Native or Dephosphorylated Troponin

Mouse	EC_{50} Percentage Motility, μM		$\frac{EC_{50}\ NF}{EC_{50}\ NF.dp}$
	Non-Failing Troponin	Non-Failing Troponin. dp	
N11.30.16	0.310 ± 0.068	0.269 ± 0.07	1.15
N11.30.16	0.148 ± 0.050	0.154 ± 0.052	0.96
N11.30.12	0.090 ± 0.028	0.076 ± 0.027	1.18
N11.30.12	0.414 ± 0.126	0.332 ± 0.091	1.25
Mean ± SE (n = 4)	0.234 ± 0.072	0.187 ± 0.054	1.14 ± 0.06
Student's t-test (paired)	p = 0.16		Single group, p = 0.12 (compared with 1)
(Unpaired)	p = 0.62		

SUPPLEMENTAL DATA J

Ca^{2+} -sensitivity of thin filaments containing E99K actin from human sample 2

Ji Comparison of the Ca^{2+}- activation of thin filaments containing donor heart or E99K sample 2 actin plus donor heart troponin and tropomyosin

	Donor actin EC_{50} µmol/L	E99K actin EC_{50} µmol/L	EC_{50} E99K/ EC_{50} donor
Experiment			
I	0.50±0.01	0.48±0.05	0.96
II	0.36±0.08	0.27±0.14	0.76
III	0.41±0.02	0.35±0.06	0.84
IV	0.29±0.13	0.16±0.08	0.57
V	0.54±0.14	0.39±0.29	0.73
mean	0.42±0.10	0.33±0.11	0.77±0.14
	Paired t test P=0.017		Single group t test vs 1.0 P=0.025

Jii Comparison of the Ca^{2+}- activation of thin filaments containing E99K sample 2 actin plus donor tropomyosin and donor (1.5 molsPi/mol TnI) or failing heart troponin (0.3 molsPi/mol TnI).

	E99K actin Donor troponin EC_{50} µmol/L	E99K actin faiiling troponin EC_{50}µmol/L	EC_{50}donor/ EC_{50} failing
Experiment			
I	0.11±0.04	0.20±0.07	0.54
II	0.11±0.05	0.12±0.07	0.91
III	0.06±0.03	0.05±0.01	1.3
mean	0.095±0.027	0.012±0.075	0.93±0.37

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