SUPPLEMENTAL MATERIAL

Molecular and Functional Characterization of a Novel Cardiac Specific Human Tropomyosin Isoform

First author's surname: Rajan **Short Title:** Novel tropomyosin isoform in heart failure patients

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Supplemental Methods

Generation of TPM1κ transgenic mice

The transgenic construct was made using a cDNA encoding human TPM1κ cloned into a vector, which contains the cardiac-specific α-MHC promoter and the human growth hormone poly(A) signal sequence.[1] The transgene construct was purified and used to generate Tg mice by standard techniques at the University of Cincinnati using the FVB/N strain. Founder mice were identified by PCR and three lines of Tg mice with varied copy numbers of the transgene were confirmed by Southern blot analysis. Transgenic expression of TPM1κ protein in the heart was confirmed by Western blot analyses. Animal experiments were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Antibodies

Anti-TPM1κ antisera used in these studies was raised in rabbits by immunization with a unique peptide corresponding to amino acids 46–61 (KEKLLRVSEDERDRV) (exon2a) of TPM1κ,. The antisera was affinity-purified (Sigma-Genosys) and used at 1:1000 dilution. Western blot analyses using the monoclonal sarcomeric anti-tropomyosin specific antibody, CH1 (Sigma), was conducted using a 1:5000 dilution. Rabbit smooth muscle anti-tropomyosin antibody (Sigma, T-3651) was used at a at 1:500 dilution; monoclonal anti-α-sarcomeric actin antibody, 5C5 (Sigma) and anti-α-tubulin antibody, B-5-1-2 (Sigma), were both used at 1:5000 dilution.

Myofibrillar protein analyses

Myofibrillar proteins were prepared from ventricular myocardium as described[2] and Western blot analysis was conducted using the TPM1κ antibody and the striated muscle TM (CH1) and actin (5C5) antibodies. The intensity of the bands was quantified by using ImageQuant v5.1 software.

Histopathological analyses

Mouse hearts (lines 72 and 80) at different ages (3, 6 and 12 month-old) and both the sexes were analyzed (n=3 for Tg and Ntg for each time point). Heart weight-to-body weight ratios were calculated to determine if cardiac hypertrophy occurred. For histological analyses, hearts were fixed in 10% neutral buffered formalin for 48 hrs. The hearts were dehydrated through a gradient of alcohols and xylene, followed by embedding in paraffin. Step-serial sections (5 μm) were taken from the hearts and stained with hematoxylin/eosin or Masson's trichrome. An expert, who was blinded to genotype, evaluated the presence of necrosis, fibrosis, myocyte disarray and calcification. Immunohistochemical analyses were performed in paraffin-embedded tissue sections by indirect immunostaining using the TPM1κ antibody diluted at 1:200 and incubating at 37^{o}C for 1 hr.

Quantitative real-time RT-PCR analyses

First strand cDNA was synthesized for 50 min at 50°C in a 20 μl reaction containing 1x First-Strand Buffer, 5 μg total RNA, 50 ng of random hexamers or gene specific primer, 2 μmol/L dNTPs, 40 units RNase inhibitor, and 200 units Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed in a 20 μl reaction, 96 well format (0.2 μl cDNA; 250 nmol/L of forward and reverse primer; 1x DyNAmo HS SYBR Green Master mix (Finnzymes)) using an Opticon 2 real-time PCR machine (Bio Rad). Three samples were measured in each experimental group in triplicate, with a minimum of two independent experiments. The relative amount of target mRNA normalized to GAPDH was calculated according to the method described by Pfaffl.[3] Specific primers that were used for the real-time PCR amplification included:

TPM1α,	Forward primer: 5'-TGTCACTGCAAAAGAAACTC-3'
	Reverse primer: 5'-CTTCAGCATCGGTGGC-3'
TPM1κ,	Forward primer: 5'-AAGGAGAAGTTGCTGCGGGTGT-3'
	Reverse primer: 5' -ATGCGTCTGTTCAGAGAAGCTACG-3'
GAPDH,	Forward primer: 5'-CATGAGAAGTATGACAACAGCCT-3'
	Reverse primer: 5'-AGTCCTTCCACGATACCAAAGT-3'

The gene specific primer used for the first strand cDNA synthesis of the striated TPM1 mRNA was a reverse primer located at the stop codon of the TPM1 mRNA with the following sequence: 5'-ATGGAAGTCATATCGTTGAGAGCGTG -3'.

Echocardiography

Mice (two and five month-old) were anesthetized with isoflurane in 100% oxygen by face mask using the minimum concentration of isoflurane to suppress podal reflex (0.5-1.5%). Body temperature was monitored with a rectal thermometer and maintained at 36-37^{o}C with a heating pad and lamp. The heart rate and ECG were continuously monitored. To obtain venous access for IV infusion of dobutamine, the right femoral vein was isolated, the distal end tied off and the proximal end catheterized with stretched PE-10 tubing. This tubing was connected to a 250 μL glass syringe mounted on a Model 355 micro infusion pump (Sage Instruments, Cambridge, MA).

Echocardiography was performed using a Vevo 770 High-Resolution *in vivo* Imaging System and RMVTM 707B "high frame" scan head with a center frequency of 30 MHz (VisualSonics, Toronto, ON, Canada). Images were acquired at baseline conditions and after infusion of dobutamine (8 and 32 ng.g body wt^{-1}.min^{-1}). AM Mode images of the left ventricle (LV) outflow tract (LVOT), ascending aorta (AO) and left atrium (LA) were taken from the parasternal long axis view. The parasternal short axis view at the level of the papillary muscles was used to measure the LV internal dimension (LVID), inter-ventricular septum (IVS) and posterior wall (PW) thicknesses. Pulsed Doppler was performed with the apical four-chamber view. The mitral inflow was recorded with the Doppler sample volume at the tip level of the mitral valve leaflets to obtain the peak velocities of flow in the early phase of diastole (E) and after LA contraction (A). Then, the Doppler sample volume was moved toward the LVOT and both the mitral inflow and LV outflow were simultaneously recorded to measure the isovolumic relaxation time (IVRT). Additional information about the diastolic function was obtained with tissue Doppler imaging (TDI). Peak myocardial velocities in the early phase of diastole (E') and after LA contraction (A') were obtained with the sample volume at the septal side of the mitral annulus in the four chamber view. All measurements and calculations were averaged from 3 consecutive cycles as previously described[4] and

performed according to the American Society of Echocardiography guidelines.[5, 6] Data analysis was performed offline with the Vevo 770 Analytic Software.

Isolated anterograde perfused heart preparation

Five month-old Tg mice along with the age matched Ntg littermate controls were used. Control and transgenic (moderate copy, line 80 and high copy, line 72) mice were anesthetized through intraperitoneal injection with 100 mg/kg sodium pentobarbital and 1.5U heparin to prevent intracoronary micro thrombi. Anterograde work-performing perfusion was initiated at a workload of 250 mmHg mL/min as described.[7] Heart rate (HR), left ventricular pressure (LVP), and the mean coronary perfusion pressure were continuously monitored. The pressure curve was used to calculate the rate of pressure development (+dP/dt) and decline (-dP/dt), time to peak pressure (TPP) and time to half relaxation (RT½) using the software "Origin" (Ver. 4.0, Microcal Software, Inc). Isoproterenol was added to the perfusion fluid close to the heart at increasing concentrations ($8x10^{-11}$ to $8x10^{-7}$M) with a multispeed, microperfusion pump (model 600, Harvard Apparatus). Individual points were recorded and summarized as means ± SD.

Measurements of Ca^{2+}-dependent activation of force

Fiber bundles dissected from papillary muscles of five month-old Ntg and TPM1κ Tg hearts (line 72) were detergent-extracted (skinned) in high relaxing (HR) buffer containing 1% Triton X-100. HR buffer contained 10 mM EGTA, 41.89 mM potassium propionate, 100 mM BES, 6.75 mM $MgCl_2$, 6.22 mM Na_2ATP, 10 mM creatine phosphate and 5 mM sodium azide. The skinned fiber bundles were mounted between a force transducer and micromanipulator with cellulose-acetate glue. The sarcomere length was adjusted at 2.0 μm using laser diffraction patterns and isometric tension was recorded on a chart recorder.[8] Initially, the myofilaments were incubated in HR and then were subjected to sequential pCa solutions (8.0 - 4.5). Myosin S1 (6 μm), isolated from rabbit fast skeletal muscle and modified with N-ethylmaleimide (NEM), was then added to the HR bath and the skinned fiber bundles were incubated for 15 min.[9] After incubation, another round of pCa activation was performed. Solutions of varying pCa values were prepared by mixing HR with HR at pCa 4.5.

The following protease inhibitors were added to all solutions: pepstatin A (2.5 μg/ml), leupeptin (1 μg/ml), and phenylmethysulfonyl floride (PMSF) (50 μmol/L). All experiments were carried out at 22^{o}C, and data are presented as means ± SD.

Bacterial recombinant protein expression

Both TPM1α and TPM1κ cDNA constructs were designed to include an N-terminal Ala-Ser dipeptide, added to functionally compensate for lack of acetylation of bacterially expressed tropomyosin.[10] The recombinant tropomyosin was expressed and purified using the Champion pET SUMO Expression System (Invitrogen). In brief, the cDNA constructs were cloned into pET SUMO vector and transformed into chemically competent Mach1-T1^{R} *E. coli* according to the manufacturer's specifications. The coding sequences of the expression plasmids were confirmed by automated DNA sequencing. The plasmid DNA construct was then transformed into BL21 (DE3) One Shot *E. coli* and induced by IPTG. The recombinant tropomyosin was then purified using the ProBond resin precharged with Ni^{2+} ions. The N-terminal peptide containing the 6-His tag and SUMO fusion protein was removed employing SUMO protease.

Circular dichroism measurements

Thermal stability measurements were made by following the ellipticity (θ) of TM at 222 nm as a function of temperature, beginning at 5°C in 0.5 mol/L NaCl, 10 mmol/L sodium phosphate pH 7.5, 1 mmol/L EDTA, and 0.5 mmol/L DTT using an Aviv model 215 spectropolarimeter. Data were obtained at 2°C intervals with a protein concentration of 3 μmol/L. The apparent melting temperature and the thermodynamic parameters for TM unfolding were calculated based on the assumption that the unfolding could be fit by up to three independent helix-coil transitions with dissociation accompanying the helix-coil transition at the highest temperature, as previously described.[11]

Actin-binding assay

To determine if there are biochemical differences between TPM1κ and TPM1α proteins, we conducted *in vitro* actin-binding analysis as previously described.[12] In brief, 5 μM of cardiac F-actin was mixed with 8 different concentrations of recombinant TM protein, namely 0, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, and 3 μM in a reaction buffer containing 200 mM NaCl, 10 mM Tris-Cl (pH 7.5), 2 mM $MgCl_2$ and 0.5 mM DTT. The reaction mixtures were incubated for 60 min at room temperature, followed by ultracentrifugation at 95,000 g, and the pellet compositions were then analyzed on 10% SDS-polyacrylamide gels stained with Coomassie Blue. To monitor the influence of troponin on the actin-binding, the cosedimentation experiments were also repeated with the addition of 2 μM human cardiac troponin complex (troponin C, troponin I and troponin T) in the reaction buffer.

Structural modeling and analysis

Structural models of TPM1 κ/κ and κ/α isoforms were built using the crystal structure of TPM1 α/α (protein data bank entry: 1IC2) as a template.[13] The 1IC2 structure contains residues 1-79 for chain A and 1-77 for chain B. To create the κ isoform, substitutions were introduced using the mutagenesis wizard in the program Pymol, and choosing rotamers that maintained interhelical contacts while minimizing steric clashes. Energetic analysis was carried out using the FoldX server.[14]

Supplemental Table 1. Clinical data of the human samples used.

Parameters	Control	End stage heart failure	DCM
n	3	5	5
Age (years)	19, 52, 63	47, 52, 58 / 39, 59	56, 62, 47, 27, 29
Etiology	BD	3 ICM / 2 DCM	DCM
LV EF	65%	20%	
Medications	NA	M, D, C, L	Diuretics, ACE inhibitors and digitalis glycosides

BD, brain death; DHD, donor heart dysfunction; ICM, ischemic cardiomyopathy; DCM, idiopathic dilated cardiomyopathy; LV EF, left ventricular ejection fraction; M, milrinone; D, digoxin; C, carvedilol; L, lisinopril.;

n, number of samples; NA, not available.

Supplemental Table 2. Body weight and baseline echocardiographic parameters.

Parameters	NTG		Tg L72	
	2 mo (7)	5 mo (6)	2 mo (7)	5 mo (4)
Body weight (g)	24.1 ± 0.97	25.6 ± 1.79	23.1 ± 1.28	27.4 ± 1.63
LA (mm)	1.88 ± 0.02	1.86 ± 0.04	1.93 ± 0.02	1.93 ± 0.10
LVIDd (mm)	3.86 ± 0.05	3.70 ± 0.10	4.19 ± 0.11*	4.26 ± 0.10*
LVIDs (mm)	2.44 ± 0.07	2.52 ± 0.09	3.06 ± 0.14*	3.10 ± 0.27*
IVS (mm)	0.66 ± 0.02	0.77 ± 0.02^{+}	0.66 ± 0.02	0.75 ± 0.01^{+}
PW (mm)	0.71 ± 0.02	0.77 ± 0.01^{+}	0.67 ± 0.01	0.73 ± 0.01
LV mass (mg)	72.7 ± 3.47	80.2 ± 3.96	80.2 ± 5.79	92.7 ± 7.10
FS (%)	36.9 ± 2.04	35.3 ± 1.57	28.3 ± 0.96*	24.3 ± 2.24*
EF(%)	67.1 ± 2.59	65.5 ± 2.08	55.1 ± 1.50*	48.6 ± 3.98*
SV (µL)	40.7 ± 4.10	34.8 ± 2.35	37.1 ± 2.87	32.6 ± 2.85
CO (mL/min)	16.7 ± 1.16	15.6 ± 1.43	15.6 ± 1.16	15.4 ± 1.22
E/A ratio	1.61 ± 0.07	1.57 ± 0.17	1.77 ± 0.14	1.27 ± 0.11^{+}
IVRT (ms)	14.3 ± 0.37	15.0 ± 0.32	17.7 ± 0.42*	17.3 ± 0.91*
E wave DT (ms)	25.5 ± 1.70	24.6 ± 1.31	28.0 ± 2.24	23.1 ± 1.48
E' (mm/s)	24.3 ± 1.11	23.6 ± 2.36	19.7 ± 0.87*	19.2 ± 2.15*
A' (mm/s)	21.3 ± 1.04	19.1 ± 1.41	18.6 ± 0.72	20.8 ± 3.09
E'/A' ratio	1.14 ± 0.07	1.28 ± 0.17	1.06± 0.06	0.95 ± 0.11
E/E' ratio	42.3 ± 1.94	44.6 ± 4.49	47.7 ± 2.48	43.4 ± 6.61

LA, left atrial internal dimension; LV, left ventricle; LVIDd, end-diastolic LV internal dimension; LVIDs, end-systolic LV internal dimension; IVS, inter-ventricular septum; PW, posterior wall; FS, fractional shortening; SV, stroke volume; CO, cardiac output; E, peak velocity of mitral inflow in the early phase of diastole; A, peak velocity of mitral inflow after LA contraction; IVRT, isovolumic relaxation time; E wave DT, desaceleration time of the E wave; E', peak myocardial velocity in the early phase of diastole; A', peak myocardial velocity after LA contraction.

Statistical analysis: Two way ANOVA (measurements were done in different ages and different groups of animals); $P<0.05$, * Significant when compared to NTG with the same age; + Significant when compared to two month-old in the same group.

Supplemental Table 3. Heart rate and systolic LV function before and after dobutamine administration.

	2 month old					
	NTG (n=7)			Tg L72 (n=7)		
	Baseline	**8 ng.g.min^{-1}**	**32 ng.g.min^{-1}**	**Baseline**	**8 ng.g.min^{-1}**	**32 ng.g.min^{-1}**
Heart Rate (bpm)	409.6 ± 23.5	436.6 ± 16.3	466.7 ± 19.1$^{+\#}$	421.1 ± 7.24	444.1 ± 7.24	468.1 ± 7.74 $^{+}$
FS (%)	36.9 ± 2.04	38.3 ± 0.50	44.1 ± 3.44 $^{+}$	28.3 ± 0.96*	31.0 ± 1.45*	33.4 ± 0.90*
EF(%)	67.1 ± 2.59	69.35± 0.66	75.2 ± 3.98 $^{+}$	55.1 ± 1.50*	59.1 ± 2.15*	62.8 ± 1.36*
SV (µL)	40.7 ± 4.10	41.7 ± 3.29	45.24 ± 3.13	37.1 ± 2.87	37.6 ± 2.29	37.3 ± 2.61
CO (mL/min)	16.7 ± 1.16	17.6 ± 1.51	18.5 ± 1.81	15.6 ± 1.16	17.0 ± 1.28	16.6 ± 1.27
	5 month old					
	NTG (n=6)			Tg L72 (n=4)		
	Baseline	**8 ng.g.min^{-1}**	**32 ng.g.min^{-1}**	**Baseline**	**8 ng.g.min^{-1}**	**32 ng.g.min^{-1}**
Heart Rate (bpm)	452.7 ± 35.8	476.3 ± 38.0^{+}	502.0 ± 39.0 $^{+\#}$	475.5 ± 26.4	503.3 ± 31.8^{+}	516.0 ± 35.2^{+}
FS (%)	35.3 ± 1.57	42.7 ± 2.23^{+}	49.2 ± 3.7^{+}	24.3 ± 2.24*	27.8 ± 2.25*	34.3 ± 3.4*$^{+}$
EF(%)	65.5 ± 2.08	74.6 ± 2.59^{+}	80.7 ± 3.6^{+}	48.6 ± 3.98*	54.3 ± 3.49*	63.5 ± 4.9*$^{+}$
SV (µL)	34.8 ± 2.35	34.7 ± 2.41	40.3 ± 2.5	32.6 ± 2.85	35.4 ± 2.84	42.4 ± 2.4
CO (mL/min)	15.6 ± 1.43	16.3 ± 1.07	20.5 ± 1.5^{+}	15.4 ± 1.22	17.6 ± 0.46	21.8 ± 1.6^{+}

bpm, beats per minute; FS, fractional shortening; EF, Ejection Fraction; SV, Stroke Volume; CO, Cardiac Output;

Statistical analysis: Two way repeated measures ANOVA (Measurements were done in baseline and two different concentrations of dobutamine in the same animal); $P<0.05$, * Significant when compared to NTG with the same concentration of dobutamine; $^{+}$ Significant when compared to baseline (within the group); $^{\#}$ Significant when compared to 8 ng.g.min^{-1} (within the group).

Supplemental Table 4. Cardiovascular and contractile parameters of Ntg and Tg mouse hearts in the isolated work-performing heart preparations at 5 months of age.

Parameters	Ntg n=6	Tg L80, n=5	Unpaired t test, Ntg vs. Tg	Tg L72, n=5	Unpaired t test, Ntg vs. Tg	Post hoc analysis p-value
SP, mmHg	112.6 ± 4.6	94.285 ± 2.6	**P = 0.005**	85.0 ± 4.0	**P = 0.002**	0.0074, Ntg vs. L80, L72
DP, mmHg	-10.6 ± 1.9	-5.6 ± 1.9	P = 0.087	-3.4 ± 0.6	**P = 0.032**	n/s
EDP, mmHg	3.5 ± 0.9	6.9 ± 0.39	**P = 0.006**	11.1 ± 2.1	**P = 0.048**	0.0233 Ntg vs. L72
+dP/dt, mmHg/s	4204 ± 118	3221 ± 189	**P = 0.023**	2706 ± 97	**P = 0.0001**	0.007 Ntg vs. L80, L72
-dP/dt, mmHg/s	3128 ± 169	2238 ± 128	**P = 0.007**	2049 ± 103	**P = 0.0006**	0.0059 Ntg vs. L80, L72
HR, beats/min	346 ± 5.0	349 ± 35	P = 0.32	350 ± 24	P = 0.85	n/s
TPP, ms/mmHg	0.37 ± 0.035	0.49 ± 0.026	**P = 0.025**	0.48 ± 0.025	**P = 0.038**	n/s
RT½, ms/mmHg	0.60 ± 0.028	0.68 ± 0.15	P = 0.61	0.74 ± 0.16	**P = 0.032**	n/s

SP, Systolic pressure; DP, Diastolic pressure; EDP, End diastolic pressure; +dP/dt, maximal rate of pressure development; -dP/dt, maximal rate of pressure decline; HR, Heart rate; TPP, time to peak pressure; RT½, half time to relaxation.

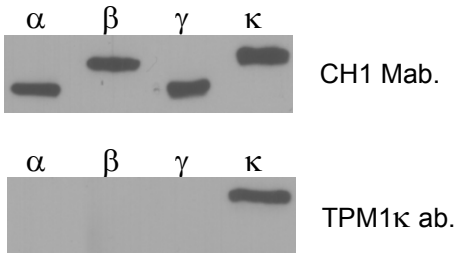
Statistical analysis: Unpaired t test, Non-parametric Kruskal Wallis test and Freidman test with post hoc analysis.

Supplemental Table 5. Energetic analysis of amino acid substitutions in TPM1κ.

Residue[a]	Position[b]	FoldX ΔΔG[c]	HΦ[d]	SB[e]	Notes
D41E	*f*				
E42D	*g*				
L43I	*a*	+	Lose		
V44A	*b*				
S45-	*c*				
L46K	*d*	++++	Lose	Add	SB only added in κ/κ, not κ/α
Q47E	*e*	–––		Add	SB only added in κ/κ, not κ/α
K48-	*f*				
K49L	*g*	+		Lose	Lose SB with E54 (mutated in DCM)
L50-	*a*				
K51R	*b*				
G52V	*c*				
T53S	*d*	++	Lose		Lesser effect in κ/α
E54-	*e*				
D55-	*f*				
E56-	*g*				
L57R	*a*	+++	Lose	Add	Add bidentate SB
D58-	*b*				
K59R	*c*				
Y60V	*d*	++	Lose		Lesser effect in κ/α
S61L	*e*	–––			FoldX predicts more favorable folding
E62-	*f*				
A63E	*g*	+			
L64-	*a*				
K65H	*b*				
D66K	*c*				
A67-	*d*				
Q68E	*e*	++			Electrostatic clash with E63
E69D	*f*				
K70S	*g*	++			Less favorable electrostatics and packing
L71-	*a*				
E72L	*b*				
L73A	*c*				
A74-	*d*				
E75-	*e*				
K76-	*f*				
K77A	*g*	–	Lose		Lesser effect in κ/α
A78-	*a*				
T79A	*b*				
D80K	*c*				

[a] Residues identical in the α and κ isoforms are shown by a dash after the residue number.
[b] "Position" refers to the location in the leucine zipper coiled-coil motif; *a* and *d* are interface residues; *e* and *g* are outer residues that may interact across the coiled coil; *b*, *c*, and *f* are on the far side from the coiled-coil interface.
[c] FoldX ΔΔG refers to the predicted stability of the κ/κ coiled coil relative to the α/α coiled coil. The symbols and colors indicate the degree of destabilization (yellow/orange) or stabilization (blue) relative to α/α. The symbols are as follows: Destabilizing substitutions, +: ΔΔG = 0 to +0.5 kcal/mol; ++: ΔΔG = +0.5 to 1.0 kcal/mol; +++: ΔΔG = +1.0 to 1.5 kcal/mol; ++++: ΔΔG = >1.5 kcal/mol. Stabilizing substitutions, –: ΔΔG = 0 to –0.5 kcal/mol; ––: ΔΔG = -0.5 to –1.0 kcal/mol; –––: ΔΔG = <-1.5 kcal/mol.
[d] HΦ refers to hydrophobic contacts within the coiled-coil interface.
[e] SB refers to salt bridges across the coiled-coil interface.

Supplemental Figure 1.



Supplemental Figure Legend

Western blots of recombinant TM isoforms using striated muscle TM-specific antibody (CH1) (upper panel) and a TPM1κ specific antibody (lower panel). The mobility of β-TM and TPM1κ is switched in the presence and absence of SDS in the gel. The above supplemental figure shows separation on a custom made 10% SDS-PAGE (TPM1κ is higher than β-TM). Figures 1B and C of the manuscript show TM isoform separation using BIORAD's 10% Criterion Tris-HCl precast gel (without SDS) (β-TM is higher than TPM1κ.

Supplemental Methods References

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