Supplemental Material

Expanded Methods

Transcript and Protein Analysis

Isolation of mRNA was performed using the Rneasy kit (Qiagen) and reverse transcription was performed with Omniscript (Qiagen). Quantitative RT-PCR was performed using validated Taqman® (Life Technologies) assays, except for the nuclear isoform transcript of CAMK2D (custom Taqman® assay) and RCAN 1.2 (SYBR® Green assay) (see Supplemental Table 1). On target PCR amplification of the PCR product was validated for both the latter assays using gel electrophoresis, DNA extraction, and Sanger sequencing.

Protein immunoblotting was performed using standard methods. All blots were blocked with 5% milk and incubated overnight unless otherwise stated. The antibodies and specific conditions are as follows: SERCA2 (Thermo Pierce MA3-919, 1:1000), PLN (Millipore 05-205, 1:1000), Thr17-phospho-PLN (Badrilla, UK A010-13, 1:5000), Ser16-phospho-phospholamban (Badrilla, UK A010-12, 1:5000), $Ca_v1.2$ (Alomone Labs, Israel ACC-003, 1:500, blocked with 5% nonfat milk in PBS, 0.3% Tween, 0.05% NaN3), NCX1 (Thermo Pierce MA3-926, 1:1000), and CSQ2 (Thermo Pierce PA1-913, 1:1000).

Primary antibodies were visualized with a secondary goat anti-rabbit antibody or goat anti-mouse antibody (Licor) diluted 1:5000 for 45 minutes. Imaging and densitometry

analyses were performed using the LI-COR5 Odyssey laser scanner and band intensity was normalized to GAPDH as a protein loading control.

SERCA Uptake Assay

SERCA Ca^{2+} uptake was measured as previously described.[1] In brief, 50 mg of frozen human heart tissue was homogenized in 750 µl of buffer containing 50 mM KH_2PO_4, 10 mM NaF, 1 mM EDTA, 300 mM sucrose, 0.3 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol (pH 7.0). Additional buffer was added to achieve a uniform protein concentration of 5µg/µl. Sodium azide inhibited Ca^{2+} uptake by the mitochondria, whereas ruthenium red and procaine inhibited Ca^{2+} release from the SR. Ca^{2+} uptake was measured over a range of pCa values from 8 to 5 in uptake buffers containing potassium oxalate as a quantitative marker for SR vesicles. After a 2-min pre-incubation of the reaction mixture containing 150 µCi/ml ^{45}Ca at 37°C, 75 µg of ventricular homogenate were added. At exactly 2 min at 37°C, the reaction was stopped by filtration through a 45-µm Millipore filter and washed with a cold buffer containing 20 mM Tris and 2 mM EGTA (pH 7.0). The SERCA Ca^{2+} uptake velocity was calculated by determining the amount of ^{45}Ca bound to the Millipore filters divided by 2 min. Total SERCA uptake capacity at each pCa was also determined by allowing reactions to continue for 1 hour at 37°C before filtering. Each measurement was performed in triplicate. Reactions were repeated for a subset of samples without phenylmethylsulfonyl fluoride and with 2.7 units/ul lambda phosphatase (NEB). Phosphatase was incubated with samples on ice for 15 minutes since incubation at

room temperature or at 37 C, as recommended by the manufacturer to achieve complete dephosphorylation, substantially reduces signal from the assay.

[^{3}H]Ryanodine Binding

[^{3}H]Ryanodine binding to cell lysates was performed as previously described.[2] To maximize signal from limited quantity of surgical myectomy tissue, total binding was determined at the optimal calcium concentration (pCa 5) to reflect maximal ryanodine activity. A binding mixture of 300 µl contained 30 µl of lysate (125 µg protein), plus a standard mixture of 200 mM KCl, 25 mM Tris/50 mM Hepes (pH 7.4), 3 mM MgATP, 1 mM EGTA, 5 nM [^{3}H]ryanodine (68.4 Ci·mmol−1, Dupont NEN, Wilmington, DE, USA), and protease inhibitors. The reaction was incubated for 2 hr at 36°C, filtered on Whatman GF/B glass filters (Whatman, Clifton, NJ, USA) presoaked with 1% polyethylenimine and washed twice with 5 mL of distilled water using a Brandel M24-R cell harvester (Gaithersburg, MD, USA). Non-specific binding was determined in the presence of 20 µM unlabelled ryanodine and was subtracted from each sample. Ryanodine modifications by nitrosylation and oxidation were determined by addition of 500 mM spermine NONOate or 5 mM H_2O_2, respectively, to the binding buffer prior to incubation. Each measurement was performed in triplicate.

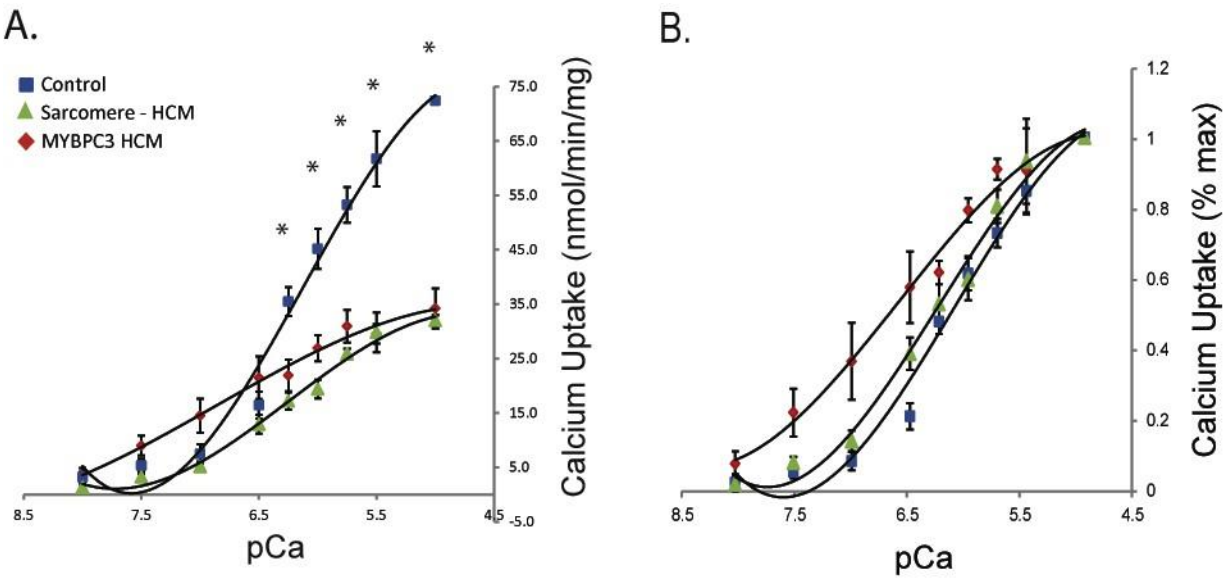
Transcription Factor Studies

Nuclear protein extraction was performed as previously described with modifications.[3] To prepare nuclei, frozen heart tissue was pulverized in liquid N_2, then resuspended in NB1 (10 mM Tris, pH 8.0, 10 mM NaCl, 3 mM MgCl2, 0.5 mM DTT, 0.1% Triton X-100,

0.1 M sucrose). The tissue was rotor-homogenized with 3 pulses of 15 seconds each on ice, gently mixed with NB2 (NB1 with added 0.25 M sucrose), then passed through a 40 µm filter. NB3 (10 mM Tris, pH 8.0, 5 mM MgCl2, 0.5 mM DTT, 0.33 M sucrose) was layered under the suspension, and the nuclei were pelleted at 1000 g for 5 min. Isolated nuclei were re-suspended in 25 mM HEPES, pH 7.8, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, and protease inhibitors, and slowly rotated at 4 °C for 30 min. Samples were then centrifuged at 4 °C in a tabletop centrifuge at maximum speed. The supernatant was transferred to a clean tube to be used for the transcription factor assays.

Activation of MEF2 was assessed by an ELISA-based method per manufacturer's recommended conditions (Active Motif, Carlsbad, CA). 10 µg of nuclear extract was added to wells coated with oligonucleotides containing the transcription factor consensus binding site and incubated for one hour on a plate shaker. Wells were treated with primary antibody (MEF2) for one hour, followed by an HRP-conjugated secondary antibody. Quantification was performed using a microplate reader at 450 hm with a reference wavelength of 655 hm. C2C12 nuclear extract was used as a positive control. Activation of NFATc1 was assessed by a similar ELISA-based method (Active Motif, Carlsbad, CA). Nuclear extract from Jurkat T-cells served as a positive control.

Supplemental Figure 1.



Supplemental Figure 1 Legend:

A. SERCA uptake velocity measured by ^{45}Ca SERCA uptake assay following pre-treatment with lambda phosphatase and in the absence of phosphatase inhibitor (control n=3, *MYBPC3*-mutation HCM n=3, sarcomere-negative HCM n=3). B. Normalized SERCA uptake velocity following phosphatase pre-treatment. *p<0.05 for controls vs. all HCM using the Mann-Whitney test with exact p-value calculation for small sample sizes.

Supplemental Table 1

Subject	Source of Tissue	DNA Mutation	Protein Mutation	Age	Gender	MWT (mm)	EF	Rest Grad	CCB	BB	NYHA Class	Septal Anatomy	Fibrosis on MRI	NSVT	CaMKII Activation	SERCA2 Reduced	SERCA Uptake Reduced
No Sarcomere Mutation																	
1	Myectomy			59	M	19	68	44	No	Yes	3	sigmoidal	No	No	No	Yes	Yes
2	Myectomy			62	M	26	75	29	Yes	Yes	2	reverse	-	Yes	No	Yes	
3	Myectomy			26	M	20	60	38	No	Yes	3	reverse	Yes	No	No	Yes	Yes
4	Myectomy			47	F	16	70	105	No	Yes	4	neutral	-	No	No	Yes	
5	Myectomy			41	M	23	66	91	No	Yes	1	reverse	Yes	No		Yes	
6	Myectomy			52	M	20	72	200	No	No	3	reverse	No	No	Borderline	Yes	Yes
7	Myectomy			51	M	22	65	15	No	Yes	2	reverse	Yes	Yes	No	Yes	
8	Myectomy			58	M	20	65	83	No	Yes	1	neutral	No	Yes		Yes	Yes
9	Myectomy			47	M	18	60	25	Yes	Yes	2	sigmoidal	No	No		Yes	
10	Myectomy			43	F	20	65	98	No	Yes	2	sigmoidal	No	No	No	Yes	Yes
***MYBPC3* Mutation**																	
11	Myectomy	**1484G>A**	**Arg495Gln**	27	F	24	60	60	No	Yes	2	NA	-	No	Yes	Yes	
12	Myectomy	**3294 G>A**	**W1098***	33	M	36	76	112	No	Yes	3	reverse	Yes	Yes		Yes	Yes
13	Myectomy	**3330+2 T>G**	**(splice)**	40	M	41	65	72	No	Yes	3	reverse	-	Yes		Yes	Yes
14	Myectomy	**2308 G>A**	**(splice)**	34	M	24	76	27	No	Yes	1	reverse	No	Yes		Yes	Yes
15	Myectomy	**1624+4 A>T**	**(splice)**	43	M	23	80	21	Yes	Yes	2	reverse	-	Yes	Yes	Yes	Yes
16	Myectomy	**1484G>A**	**Arg495Gln**	*10*	*F*	45	80	50	Yes	No	2	reverse	Yes	No	Yes	Yes	Yes
17	Myectomy	**3697 C>T**	**Gln1233***	39	F	24	75	49	No	Yes	1	reverse	Yes	No	Yes	Yes	
18	Myectomy	**1624G>C**	**Glu542Gln**	47	M	18	70	21	No	Yes	3	reverse	-	Yes		Yes	Yes
19	Myectomy	**2670 G>A**	**Trp890***	41	F	17	70	111	No	Yes	3	neutral	-	Yes	Yes	Yes	Yes
20	Myectomy	**2905 + 1G>A**	**(splice)**	48	F	19	80	163	No	Yes	2	reverse	No	Yes		Yes	
21	Myectomy	**3742_ 3759dup**	**Gly1248_ Cys1253dup**	43	F	21	75	74	No	Yes	3	reverse	Yes	No		Yes	
22	Myectomy	**772 G>A**	**(splice)**	28	M	21	70	182	Yes	No	2	sigmoidal	Yes	No	Yes	Yes	
23	Myectomy	**772 G>A**	**(splice)**	43	F	30	83	63	No	Yes	1	reverse	Yes	Yes	Yes	Yes	
24	Myectomy	**1928-2 A>G**	**(splice)**	57	F	19	72	94	No	Yes	2	sigmoid	-	No	Yes	Yes	
25	Myectomy	**927-9 G>A**	**(splice)**	59	M	23	70	44	No	Yes	3	reverse	-	Yes	Yes	Yes	
26	Myectomy	**1624+4 A>T**	**(splice)**	31	F	21	70	97	No	Yes	1	neutral	No	No		Yes	
27	Myectomy	**3233 G>A**	**Trp1078***	35	F	21	76	64	Yes	No	2	reverse	No	No	Yes	Yes	
***MYH7* Mutation**																	
29	Myectomy	**3286 G>T**	**Asp1096Tyr**	52	M	18	65	38	No	Yes	3	sigmoid	No	No	Yes	Yes	Yes

30	Myectomy	**5135G>A**	**Arg1712Gln**	36	M	17	75	86	Yes	Yes	2	reverse	No	No		Yes	Yes
31	Myectomy	**4816 C>T**	**Arg1606Cys**	59	M	20	80	90	No	Yes	3	sigmoid	No	No	Borderline	Yes	
32	Myectomy	**968 T>A**	**Ile323Asn**	12	M	23	91	82	No	No	1	NA	Yes	Yes		Yes	
33	Myectomy	**1370 T>C**	**Ile457Thr**	72	F	16	75	39	No	No	3	neutral	Yes	No	No	Yes	Yes
34	Myectomy	**1816 G>A**	**Val606Met**	39	F	17	75	70	No	Yes	3	reverse	Yes	No		Yes	
35	Surgical explant	**4130C>T**	**Thr1377Met**	45	F	16	60	-	No	Yes	3	neutral	-	Yes	Yes	Yes	
36	Surgical explant	**2123G>C**	**Gly708Ala**	36	F	19	60	-	No	No	4	reverse	-	Yes	Yes	Yes	

MWT = maximal wall thickness. EF = left ventricular ejection fraction. Rest Grad = resting left ventricular outflow tract gradient (mm Hg). CCB = calcium channel blocker prescription. BB = beta blocker prescription. NYHA Class = New York Heart Association congestive heart failure symptom class. Septal anatomy = morphology of left ventricular septal hypertrophy. Reverse = reverse curvature. NSVT = nonsustained ventricular tachycardia. *CaMKII activation defined as at least 1.5-fold upregulation of pT287-CaMKII ("Borderline" if at least 1.3-fold upregulation). ^SERCA2 reduced defined as a reduction in SERCA2 mRNA or protein abundance by at least 20% compared to average of controls. +SERCA uptake reduced defined as a reduction in SERCA uptake velocity at pCa=5 of at least 20% of average of controls.

Supplemental Table 2.

Gene	*qRT-PCR Assay*	*Assay #*	*Primers*
ATP2A2 (SERCA2)	**Taqman**	Hs00544877_m1	**-**
CACNA1C (L-type calcium channel)	**Taqman**	Hs00167681_m1	**-**
CAMK2D	**Taqman**	Hs00943550_m1	**-**
CAMK2D Nuclear	**Taqman**		
CAMK2G	**Taqman**	Hs00538454_m1	**-**
CASQ2 (calsequestrin)	**Taqman**	Hs00154286_m1	**-**
MEF2A	**Taqman**	Hs01050409_m1	**-**
MEF2C	**Taqman**	Hs00231149_m1	**-**
MEF2D	**Taqman**	Hs00954735_m1	**-**
PLN	**Taqman**	Hs00160179_m1	

(phospholamban)			
PPP3CB (Calcineurin, catalytic subunit)	**Taqman**	Hs00917458_m1	**-**
RCAN 1.2	**Sybr Green**	-	GCTCCGCCAAATCCAGACAA GCTGCGTGCAATTCATACTTTTC
RYR (ryanodine)	**Taqman**	Hs00892883_m1	
SLC8A1 (NCX, sodium-calcium exchanger)	**Taqman**	Hs01062258_m1	**-**

References

1. Guinto, P.J., Haim, T.E., Dowell-Martino, C.C., Sibinga, N. & Tardiff, J.C. Temporal and mutation-specific alterations in Ca2+ homeostasis differentially determine the progression of cTnT-related cardiomyopathies in murine models. *Am J Physiol Heart Circ Physiol* 297:H614-626.
2. Loaiza, R.*, et al.* Heterogeneity of ryanodine receptor dysfunction in a mouse model of catecholaminergic polymorphic ventricular tachycardia. *Circ Res* 112:298-308.
3. Phelps, D.E. & Dressler, G.R. Identification of novel Pax-2 binding sites by chromatin precipitation. *J Biol Chem* 271:7978-7985.