SUPPLEMENTAL MATERIAL (Dai, et al)

Expanded Methods

Animal Longevity Cohort

Mice (C57Bl6) were housed as previously described [1], fed regular chow diet (irradiated Picolab Rodent Diet 20 #5053, PMI Nutrition International, Brentwood, MO) and reverse osmosis water. They were kept in a barrier specific-pathogen-free facility maintained at 70-74ºF, 45-55% humidity, with 28 air changes/hour and 12/12-h light/dark cycle. Mice were selected for echocardiography using consecutive identification numbers assigned at birth. All animals in the longevity cohort were not subjected to any invasive experimentation other than echocardiography. All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

Echocardiography

A reduced dose of ketamine/xylazine (65mg/kg and 4.4 mg/kg, respectively), providing adequate sedation but minimal cardiac suppression, was used to minimize the effect of anesthesia or stress on cardiac function. The mice were placed in a lateral decubitus position on a heat-pad to maintain the body

temperature at 37°C. Transthoracic echocardiography was performed with Acuson CV-70 (Siemens) equipped with a 13 MHz-transducer. Images were collected and stored as a digital cine loop for measurements and calculations. Standard imaging planes, M-mode, conventional and Tissue Doppler, and functional calculations were performed according to American Society of Echocardiography guidelines. The parasternal long-axis view was used for the measurement of the left atrium (LA) dimension, the left ventricle (LV) wall thickness and chamber dimension. These parameters were applied to estimate the fractional shortening and LV mass. The apical long-axis view was used to obtain the pulsed-waved Doppler of mitral inflow, aortic ejection, isovolemic contraction and relaxation time as well as Tissue Doppler Imaging of the mitral annulus (Ea/Aa). MPI (Tei index) was calculated as the ratio of the sum of isovolemic contraction and relaxation time to LV ejection time [2]. For both pulsed-wave and tissue Doppler recording, a sample size of 0.2 mm was used. A sweep speed of 40 mm/s was used for M-mode and Doppler studies.

Quantitative Pathology

One day after echocardiography, the second series of 42 old mice (24 MCAT, 18 WT) aged 24-29 months were euthanized for cross-sectional study of heart

pathology. Trichrome and H&E staining was performed. Myocardial fiber width was quantified as the average fiber width of 40-50 longitudinal sections of myocytes from 4-5 high power field images per heart section (400x). Ventricular fibrosis was quantified from Trichrome stained sections, in which the percentage of blue-green area was measured relative to the total cross-sectional area of the ventricles.

The random mutation capture assay of mt DNA point mutations was performed as previously described [3,4] with slight modifications. Briefly, a crude mitochondrial fraction was isolated, followed by mtDNA purification and extensive mtDNA digestion with TaqI endonuclease. Real-time PCR was performed with two primer sets. The first primer set, mTaq634, flanks a TaqI restriction site. Termed PCR A, this sequence is amplified only if there is a mutation within the TaqI restriction site. The second primer set, termed control primers, amplifies an adjacent region not containing a TagI restriction site, is performed to quantitate the total amount of mtDNA template in the sample (called PCR B). In addition, a standard curve using control primers is generated in parallel to control for PCR efficiency and to set an independent standard for the quantification of WT and mutant mtDNA molecules. Using a

comparative PCR-strategy, WT and mutant molecules are quantified, and the amount of mutant molecules is divided by the amount of WT molecules in order to calculate the mutation frequency. This strategy requires a 100% digestion of mtDNA molecules with WT TaqI restriction sites, although rare WT molecules may persist. To correct for the presence of these rare WT mtDNA molecules, the product of PCR A was further digested using TaqI endonuclease, and the fraction of mutant, or undigested PCR-product, (250 bp, called C) vs WT or digested (150 bp, called D) was quantified using Agilent Bioanalyzer DNA-1000 microelectrophoresis (Agilent Technologies, Germany). The mtDNA mutation frequency was estimated as relative quantity of C/(C+D) x A/B/4. The sequences for mTaq634 forward and reverse primers are ACTCAAAGGACTTGGCGGTA & AGCCCATTTCTTCCC ATTTC, respectively and for control forward and reverse primers are TCGGCGTAAAACGTGTCAACT & CCGCCAA GTCCTTTGAGTTT, respectively. Detection of deletions was performed with the same method using a different set of primers flanking multiple Taq I restriction sites located several kb apart. PCR amplification does not take place unless all restriction sites are lost by a segmental deletion, which renders the DNA molecule

resistant to Taq I cleavage. The primers for deletion assay are AGGCCACCACACTCCTATTG and AATGCTAGGCGTTTGATTGG.

Measurement of mitochondrial protein carbonyl

LV tissues were homogenized in mitochondrial isolation buffer (sucrose 250mM, 1mM EGTA, 10mM HEPES, 10mM Tris-Hcl, pH7.4) using glass-grind homogenizer, then the lysates were centrifuged at 800g for 10 minutes. The supernatants were further centrifuged at 4000 g for 30 min at 4ºC. The crude mitochondrial pellets were then resuspended in small volume of isolation buffer and sonicated on ice. The mitochondrial extracts were treated with 1% streptomycin sulfate to precipitate mitochondrial nucleic acids. The enriched mitochondrial proteins (use 1μg) were assayed using protein carbonyl ELISA based on derivatization of the carbonyl group with dinitrophenylhydrazine (DNPH), followed by anti-DNPH antibody (OxiSelect protein carbonyl ELISA, Cell Biolabs, San Diego, CA).

Quantitative PCR

The quantitation of relative gene expressions was performed using Taqman Gene Expression Assays with Applied Biosystem 7900 real time PCR machine. The genes include: PGC1-α (Mm00731216), TFAM (Mm00447485), NRF-1 (Mm00447996), NRF-2 (Mm00487471), ANP (Mm01255747), BNP

(Mm 00435304) and MCIP-1 (Mm00517094). All expression assays were normalized to 18S RNA.

Mitochondrial DNA copy number was quantified using quantitative PCR of total DNA extracts from cardiac tissues. Mitochondrial DNA copies were estimated by the ratio of the amount of mitochondrial gene NADH dehydrogenase 1 (ND1) and a single-copy nuclear gene cytochrome P4501A1 (cyp1A1). Primers used were: ND1 (For: GAACGCAAAATCTTAGGGTACATACA, Rev: GCCGTATGGACCAACAATGTT, probe: 6FAM-CTACGAAAAGGCCC) and cyp1A1 (For:GACACAGTGATTGGCAGAGATC, Rev:AACGGATCTATGGTCTGACCTGT, probe: 6FAM-CTCAGCTGCCCTATCTGGAGG CCTTC)

Calcineurin assay and NFAT Electrophoretic Mobility Shift Assay (EMSA)

Measurement of calcineurin activity (phosphatase PP-2B) was performed using colorimetric calcineurin activity assay kit (Calbiochem), according to manufacturer`s protocol. For NFAT EMSA, consensus NFATc oligonucleotides (sc-2577, Santa Cruz) were labeled with [^{32}P]dATP using T4 Polynucleotide kinase (New England Biolab). Nuclear fraction of cardiac tissues was extracted

using nuclear extraction kit (Millipore), then quantified with Bradford assay. Fifteen μg of nuclear extracts were incubated at room temperature for 1 hour with 1.23 μg of poly (dI/dC), 20 mM Tris, pH 7.5, 100 mM NaCl, 1.6 mM DTT, 10% glycerol, 1mM EDTA, 5 – 10 x 10^5 cpm of [^{32}P]dATP-NFATc oligonucleotide. It was then separated in non-denaturing polyacrylamide gels (6%) at 150 V for 2-3 hrs in Tris-borate-EDTA buffer, pH 8.0. After electrophoresis, the gels were dried, and radioactive protein-DNA bands were detected by autoradiography.

Western Blot

LV tissues were homogenized in iced-cold lysis buffer containing protease and phosphatase inhibitors, then analyzed by standard western blots. Total protein (10-25 μg) were separated on 4–20% polyacrylamide gradient gels and transferred to nitrocellulose membranes, then blocked with 5% milk in Tris-buffer solution with 1% Tween-20. Membranes were incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C. The primary antibodies include: rabbit polyclonal anti-SERCA2 (Novus Biologicals), anti-NCX and GATA4 (Santa Cruz), p-GATA4 S105 (Abcam), anti phospho-ERK1/2 and anti-ERK1/2 (Chemicon), anti-phopho-phospholamban and anti-phospholamban (Millipore) as well as anti-GAPDH (Cell Signaling). The

secondary antibody was donkey anti-rabbit antibody (Thermo-Scientific). The antigen-antibody complexes were detected by the enhanced chemi-luminescence method (Thermo-scientific). The relative band density was quantified by using Image Quant ver.2.0 and reported as a ratio to GAPDH as an internal control. All samples were normalized to the same cardiac protein sample.

Calcium transients study

Ventricular cardiomyocytes were dissociated using standard enzymatic methods[5], then maintained in Dubelcco's MEM and loaded with acetoxymethyl-ester form of Fluo-4 (50uM), a calcium indicator dye, at room temperature for 30 min. Field stimulation was performed using IonOptix Myopacer (IonOptix Corp, Milton, MA, USA) stimulator at a frequency of 1 Hz. Olympus IX-70 inverted microscope coupled with IonOptix photometry system was used to collect calcium transients fluorescence signals from cardiomyocytes. The $[Ca^{2+}]_i$ was estimated using the equation: C= Kd x R / (Kd/C0+1-R)[6], where R denotes F/F0, Kd is the dissociation constant of Fluo-4 (600nM), and C0 is the resting $[Ca^{2+}]_i$ (=150nmol/L). The Ca-transient amplitude was reported as F/F0, where F0 is the diastolic fluo-4 fluorescense. The exponential fit of the Ca-transient decay was applied to estimate the

decay rate constant λ(s^{-1}). The amplitude of the Ca-transient evoked by the application of 20 mM caffeine was used as an indicator of SR Ca^{2+} content[7]. To ensure steady-state SR Ca^{2+} load, cells were subjected to a minimum of 10 preconditioning pulses (1 Hz) before caffeine was applied. Cyclopyazonic acid (5 μM) was applied to investigate the contribution of SERCA2 to the Ca-transients.

Measurement of cardiac angiotensins by mass spectrometry (MS)

LV tissues were homogenized and centrifuged at 3000g for 10 min at 4°C, then 3x volume of absolute ethanol was added to the supernatant on ice for 30 min, followed by centrifugation at 14,000rpm for 15 min at 4°C. The final supernatant containing peptides was dried and resuspended in 20 μL of buffer A (5% acetonitrile, 0.1% formic acid). The analysis was carried out on a tandem quadrupole MS/MS system (TSQ Ultra, Thermo Fisher) coupled to a HPLC (Surveyor, Thermo Fisher) with a flow splitter system. Samples were loaded on a 75μm x 250mm column packed in house with 4μm RP-C12 beads (Jupiter, Phenomenex). The following pairs of precursor>fragments were selected for the MS method: 523.8> 263.1, 400.2, 513.3, 676.3, 775.4, and 931.5 and were correspondent to the formation of y2, y3, y4, y5, y6 and y7-ions from the doubly charged species of the peptide DRVYIHPF (Ang II).

The pair 523.8>263.1 gave the strongest signal and was used for quantitation, whereas other transitions were used for confirmation. Samples were eluted in a 60min gradient from 5 to 80% of buffer B (80%acetonitrile, 0.1% formic acid). The flow rates were approximately 5μL/min and 250nL/min for loading and gradient elution, respectively. External calibration method with synthetic angiotensin II peptide was used for quantification.

Supp Table 1. Biochemical and physiological data for young and old WT and MCAT mice

	Young (5-8 months)		Old (24-28 months)		ANOVA p*
	WT	MCAT	WT	MCAT	
Body weight (g)	28± 6	29± 6	30 ± 4	31 ± 4	0.48
Systolic BP (mmHg)	102±18	107±19	96±15	102±10	0.51
Diastolic BP (mmHg)	81 ±18	85±20	74±12	75 ±13	0.63
Mean arterial pressure (mmHg)	88 ±18	91 ±20	81 ±13	83 ±12	0.52
Pulse pressure (mmHg)	21 ±5	20 ±3	21 ±5	23 ±6	0.48
Heart rate (bpm)	624±105	646±121	601±116	569±118	0.59
Glucose (mg/dL)	107±16	110±11	95±8	93±7	0.21
T-cholesterol (mg/dL)	97±28	89±21	89±30	77±11	0.37

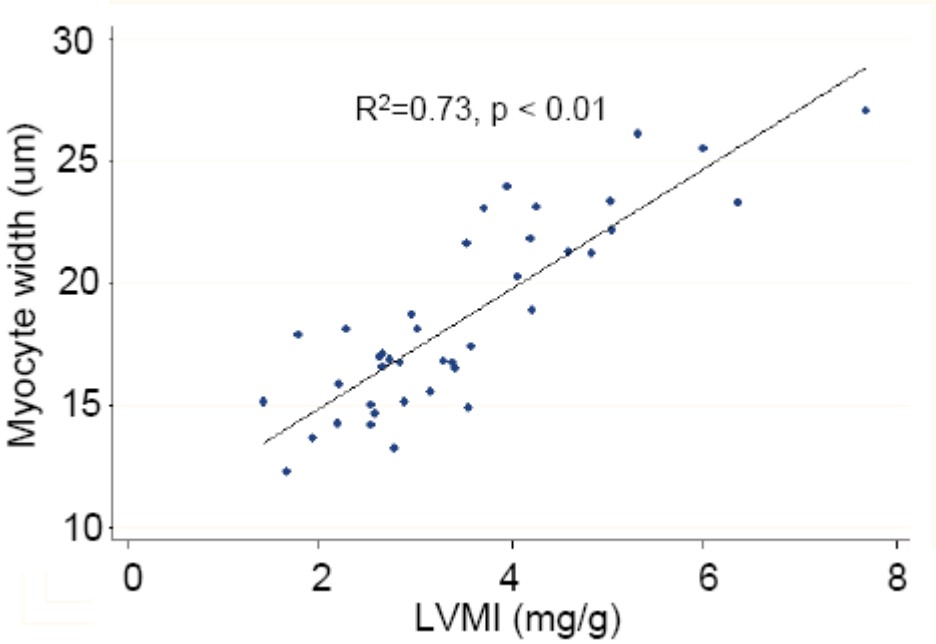
* p-values of Two-way ANOVA models including age, genotype and age*genotype interaction

Supp Table 2. Cross-sectional echocardiography in the second series of old WT and MCAT mice, and absence of differences in MCAT mice with higher vs. lower transgene expression.

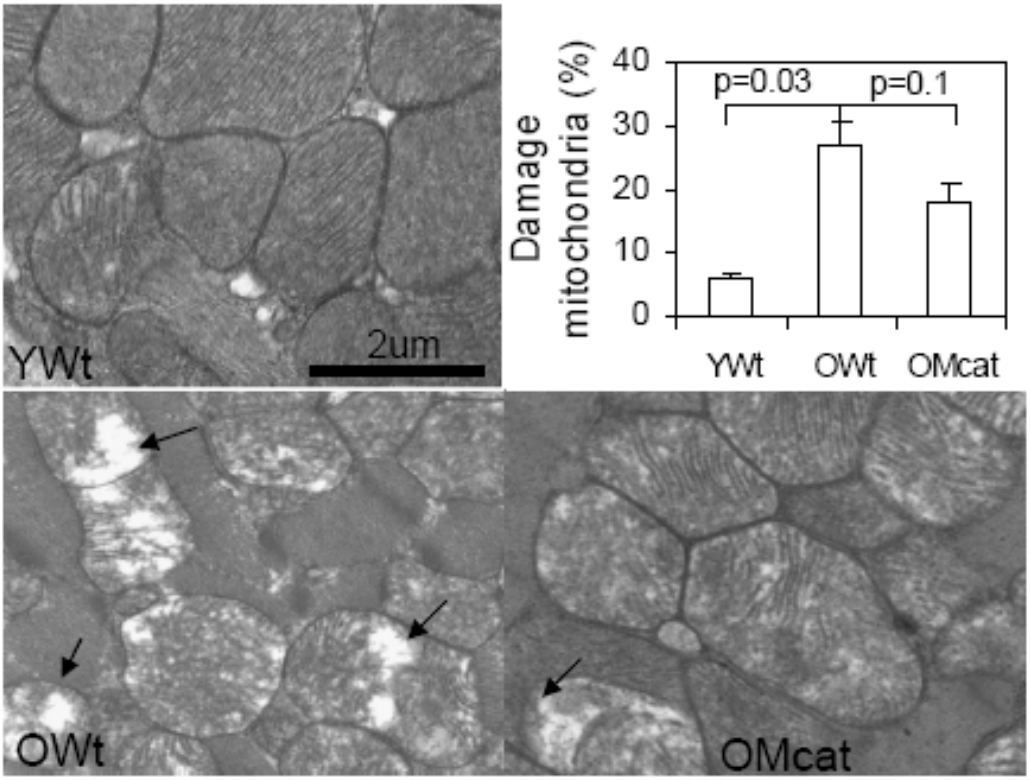
	Old WT	Old MCAT	p	Low MCAT	High MCAT
n	17	25		13	12
Age (months)	28.3 ± 0.65	27.6±0.7	0.32	28 ± 0.76	27.4 ± 0.7
Male (%)	52.9	52	0.41	53.9	50
MCAT/GAPDH mRNA (arbitrary unit)				0.5 ± 0.03	1.16 ± 0.15
Echocardiography:					
LVMI (mg/g)	4.4 ± 0.4	2.8±0.2	0.0001	3 ± 0.2	2.7 ± 0.3
LA dimension(mm)	2.0 ± 0.1	1.7±0.1	0.04	1.8 ± 0.1	1.7 ± 0.1
FS (%)	46.9± 1.1	47.8±1.1	0.55	47±1.7	48.4±1.2
Ea/Aa	0.87±0.1	1.34±0.1	0.001	1.35±0.1	1.25±0.1
Diastolic dysfunction (%)	64.8	20	0.003	23.1	16.7
MPI	0.85±0.05	0.54±0.03	0.001	0.55±0.06	0.57±0.03

Supp Table 3. Multivariate regression model predicting Ea/Aa (diastolic function)

	ß	p	95% C.I.	
SERCA2 protein	0.58	0.01	0.16	1.01
Fibrosis (%)	0.03	0.19	-0.01	0.07
Myocyte width	-0.02	0.25	-0.07	0.02



Supp Fig 1. Linear regression demonstrated that myocardial fiber width was highly correlated with LV mass index by echocardiography.



Supp Fig 2. Transmission electron micrographs of cardiac mitochondria from young Wt (4 months old), very old Wt and Mcat (36 months old), n=3 each group. Relative number of damage mitochondria was quantified blindly from 8-10 images from different fields (15000x magnification). Damage mitochondrion was defined as loss of electron density in more than 20% of the area of a mitochondrion**.**

Supp References:

1. Schriner SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS. Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science.* 2005;308:1909-11.
2. Tei C, Ling LH, Hodge DO, Bailey KR, Oh JK, Rodeheffer RJ, Tajik AJ, Seward JB. New index of combined systolic and diastolic myocardial performance: a simple and reproducible measure of cardiac function--a study in normals and dilated cardiomyopathy. *J Cardiol.* 1995;26:357-66.
3. Vermulst M, Bielas JH, Kujoth GC, Ladiges WC, Rabinovitch PS, Prolla TA, Loeb LA. Mitochondrial point mutations do not limit the natural lifespan of mice. *Nat Genet.* 2007;39:540-3.
4. Vermulst M, Wanagat J, Kujoth GC, Bielas JH, Rabinovitch PS, Prolla TA, Loeb LA. DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. *Nat Genet.* 2008;40:392-4.
5. Santana LF, Kranias EG, Lederer WJ. Calcium sparks and excitation-contraction coupling in phospholamban-deficient mouse ventricular myocytes. *J Physiol.* 1997;503 (Pt 1):21-9.
6. Song LS, Guia A, Muth JN, Rubio M, Wang SQ, Xiao RP, Josephson IR, Lakatta EG, Schwartz A, Cheng H. Ca(2+) signaling in cardiac myocytes overexpressing the alpha(1) subunit of L-type Ca(2+) channel. *Circ Res.* 2002;90:174-81.
7. Dilly KW, Rossow CF, Votaw VS, Meabon JS, Cabarrus JL, Santana LF. Mechanisms underlying variations in excitation-contraction coupling across the mouse left ventricular free wall. *J Physiol.* 2006;572:227-41.
8. Etches V, Frank J, Di Ruggiero E, Manuel D. Measuring population health: a review of indicators. *Annu Rev Public Health.* 2006;27:29-55.