Supplementary Data

Dietary Carbohydrates Restriction Inhibits the Development of Cardiac Hypertrophy and Heart Failure

Brief title: Low-Carbohydrate Diets Suppress Cardiac Hypertrophy

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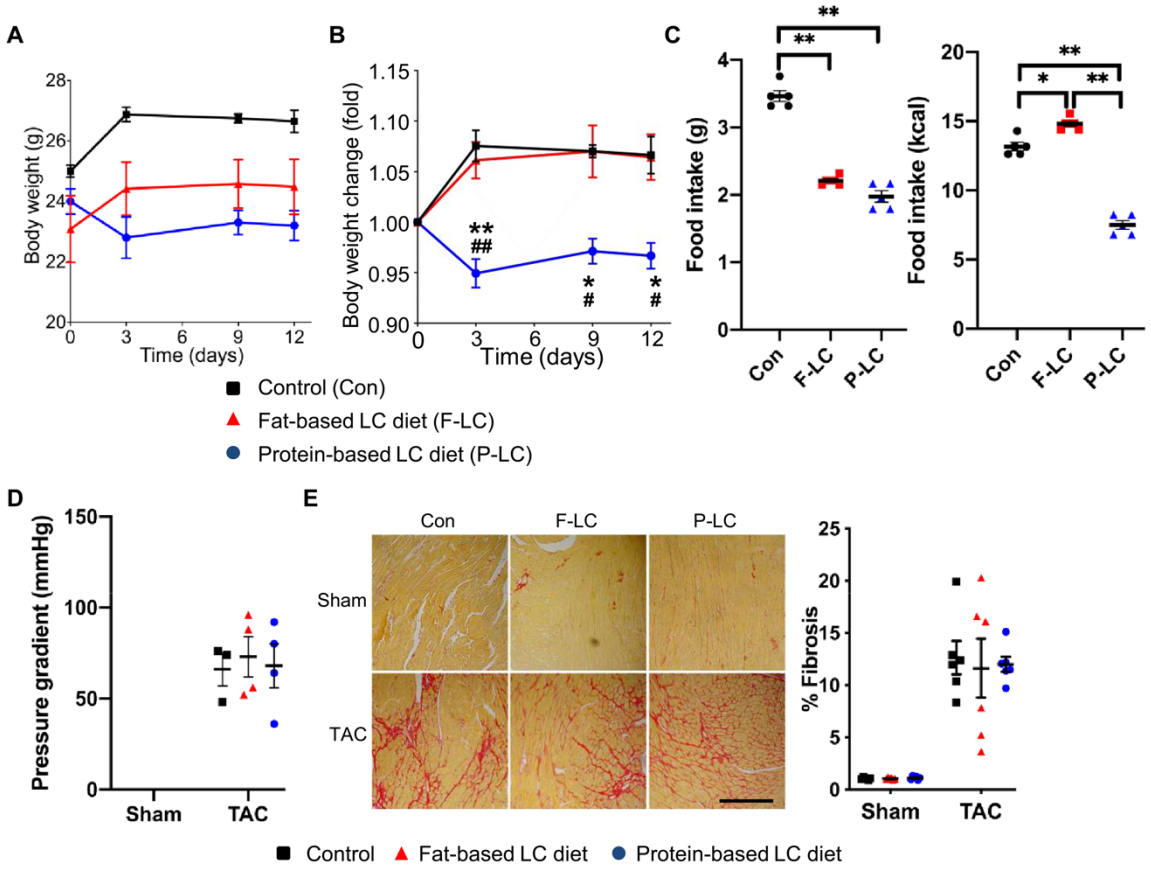
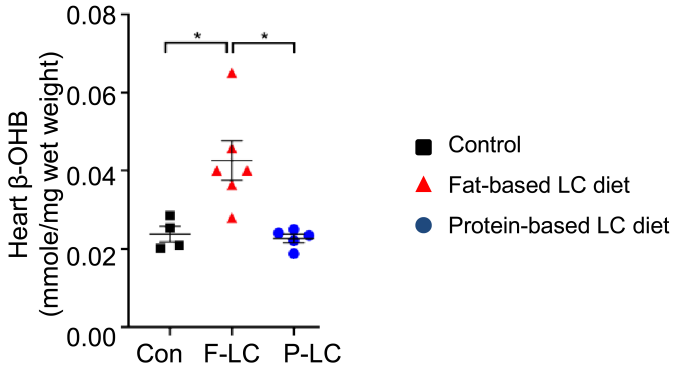
Supplementary figures and table

Supplementary Figure S1



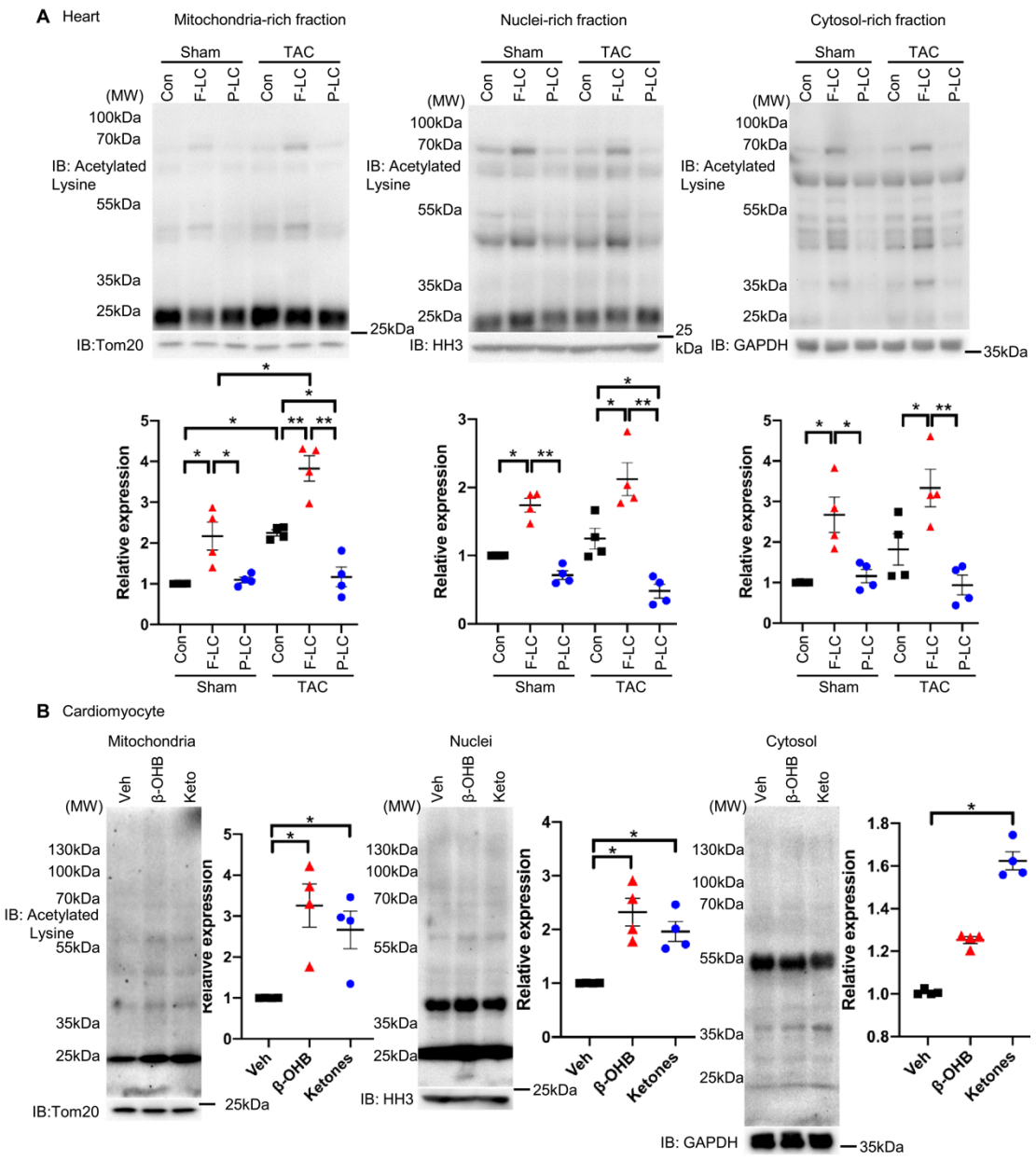
Supplementary Figure S1. Low-carbohydrate (LC) diets attenuate pressure overload-induced cardiac hypertrophy in mice. (**A**) Body weight. (**B**) Body weight change (fold change). Three groups were compared at each time point by one-way ANOVA and the Newman-Keuls multiple comparison test. Error bars indicate s.e.m. * $p < 0.05$ and ** $p < 0.001$ compared to control diet, and # $p < 0.05$ and ## $p < 0.001$ compared to fat-based LC diet (n = 4-6). (**C**) Food intake (g/day, left, and kcal/day, right) of mice fed the indicated diet (n = 5-6). Comparison by 1-way ANOVA with Tukey post hoc test. * $p < 0.05$ and ** $p < 0.001$. (**D**) Pressure gradients between the femoral artery and the ascending aorta in mice four weeks after Sham or TAC surgery. Pressure gradient was measured only in mice subjected to TAC surgery (n = 3-4). (**E**) Representative pictures of Picric Acid Sirius Red (PASR) staining, a marker of fibrosis, in heart sections of the indicated mice. Scale bar, 200 µm (left). Percentage of fibrotic area (n = 6, right). One-way ANOVA.

Supplementary Figure S2



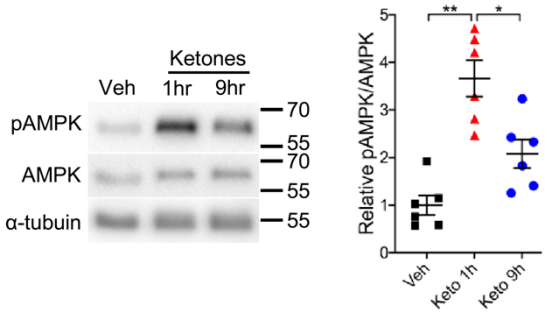
Supplementary Figure S2. Fat-based, but not protein-based, low-carbohydrate (LC) diet increases β-hydroxybutyrate (β-OHB) concentrations in the heart in mice. Myocardial level of β-OHB in C57BL/6J Wild Type mice fed the indicated diet for 10 days (n = 4-6). One-way ANOVA followed by the Tukey post-hoc analysis was used. Error bars indicate s.e.m. * $p <$ 0.05.

Supplementary Figure S3



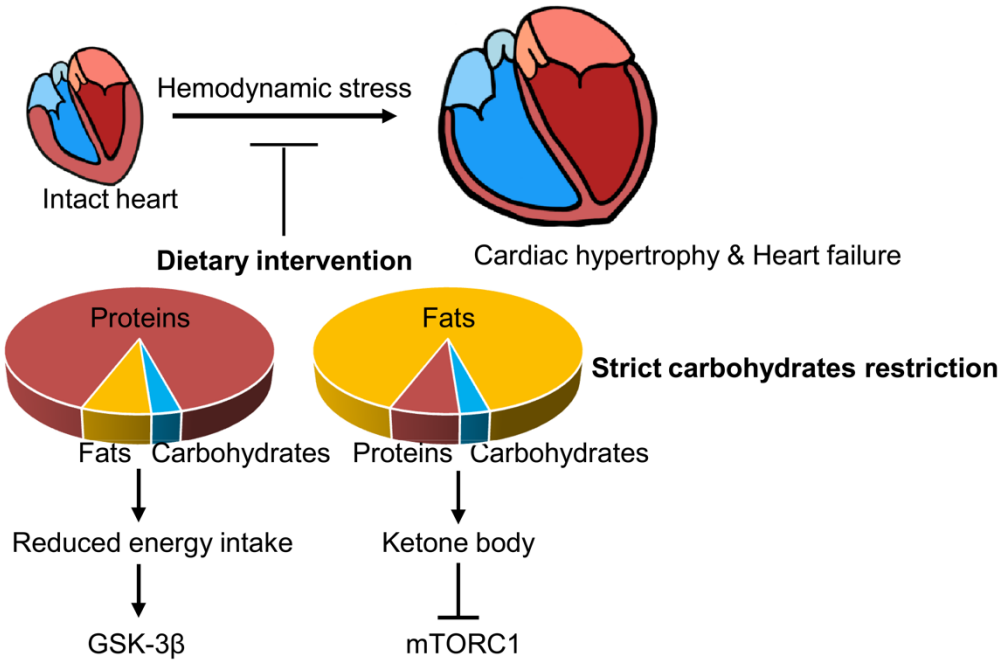
Supplementary Figure S3. Lysine acetylation is increased in mitochondrial, nuclear, and cytosolic fractions from the hearts of mice fed a fat-based low-carbohydrate diet and in cardiomyocytes treated with β-hydroxybutyrate (β-OHB) or a cocktail of ketone bodies containing β-OHB and acetoacetate. (**A-B**) Immunoblots showing lysine acetylation in each fraction isolated from hearts (A) and from cardiomyocytes (B) and their quantification analyses by 1-way ANOVA with Tukey post hoc test for normal distribution and Kruskal-Wallis test with the Dunn's multiple comparison test when the data distribution failed normality (n = 4). Error bars indicate s.e.m. * $p < 0.05$ and ** $p < 0.001$.

Supplementary Figure S4



Supplementary Figure S4. Ketone bodies increase the level of phosphorylated AMPK in cardiomyocytes. Immunoblots showing the phosphorylated (Thr172) and total levels of AMPK in cardiomyocytes treated with ketone bodies (cocktail of β-hydroxybutyrate (1 mM) and acetoacetate (500 μM)) for 1 or 9 hours. One-way ANOVA followed by the Tukey post-hoc analysis was used. Error bars indicate s.e.m. n = 6. * $p < 0.05$ and ** $p < 0.001$.

Supplementary Figure S5



Supplementary Figure S5. Schematic representation. Strict restriction of carbohydrate intake suppresses pressure overload-induced cardiac hypertrophy and heart failure. High-protein low-carbohydrate (LC) diet reduces energy intake, which decreases pressure overload-induced inhibition of GSK-3β. On the other hand, high-fat LC diet increases ketone body, which inhibits mTOR signaling.

Supplementary Table S1

	Control		**Fat-based LC**		**Protein-based LC**	
	High carbo-low sucrose		High fat-low carbo		High protein-low carbo	
	gm%	kcal%	gm%	kcal%	gm%	kcal%
Protein	19	20	12	7	85	90
Carbohydrate	67	70	5	3	2.9	3
Fat	4	10	67	90	3	7
Total		100		100		100
Kcal/gm	3.8		6.7		3.8	
Ingredient	**gm**	**kcal**	**gm**	**kcal**	**gm**	**kcal**
Casein, 80 Mesh	200	800	70	280	898.3	3593
L-Cystine	3	12	1	4	13.5	54
Corn Starch	550	2200	0	0	0	0
Maltodextrin 10	150	600	21.5	86	21.5	86
Sucrose	0	0	0	0	0	0
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard	20	180	380.2	3422	6.5	59
Mineral Mix, S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40	10	40
Vitamin Mix, V10001C	0	0	0	0	0	0
Choline Bitartrate	2	0	2	0	2	0
FD&C Yellow Dye #5	0	0	0.025	0	0.05	0
FD&C Red Dye #40	0.025	0	0.025	0	0	0
FD&C Blue Dye #1	0.025	0	0	0	0	0
Total	1055.05	4057	604.75	4057	1071.9	4057

Supplementary Table S1. Ingredients of low-carbohydrate (LC) diets supplemented with either high-fat or high-protein and high-carbohydrate control diet.

Supplementary Table S2

	SHAM			TAC		
	Con	F-LC	P-LC	Con	F-LC	P-LC
HR (/min)	466.4 ± 6.5	488.4 ± 12.7	450.2 ± 13.7	504.4 ± 20.6	489.8 ± 12.2	514 ± 21.0
IVSd (mm)	0.510 ± 0.010	0.513 ± 0.010	0.539 ± 0.024	1.043 ± 0.029	0.809 ± 0.027 ***	0.897 ± 0.034 **
LVDd (mm)	3.791 ± 0.032	3.770 ± 0.071	3.729 ± 0.067	4.543 ± 0.067	4.064 ± 0.101 ***	4.111 ± 0.071 **
LVDs (mm)	2.569 ± 0.049	2.570 ± 0.057	2.609 ± 0.034	3.593 ± 0.071	2.924 ± 0.080 ***	3.049 ± 0.062 ***
EF (%)	68.59 ± 2.23	66.57 ± 2.04	65.67 ± 1.32	50.66 ± 1.80	61.99 ± 2.09 **	59.48 ± 1.06 *

Supplementary Table S2. Echocardiographic parameters of C57BL/6J wild-type mice. Fat-based low-carbohydrate (F-LC), protein-based LC (P-LC) or control high-carbohydrate (Con) diet was fed for 4 weeks starting at day 2 after transverse aortic constriction (TAC) or sham surgery. One-way ANOVA followed by the Tukey's post-hoc analysis. Data are mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to TAC with control diet. HR, heart rate (HR); IVSd, interventricular septal wall thickness at end-diastole; LVDd, left ventricular end-diastolic diameter; LVDs, LV end-systolic diameter; and EF, ejection fraction.

Supplementary Table S3

	SHAM			TAC		
	Con	F-LC	P-LC	Con	F-LC	P-LC
HR (/min)	544.1 ± 20.8	508.3 ± 28.8	507.1 ± 19.2	477.2 ± 23.4	460.1 ± 9.7	468.5 ± 12.1
IVSd (mm)	0.493 ± 0.023	0.537 ± 0.017	0.542 ± 0.009	0.902 ± 0.025	0.947 ± 0.050	0.765 ± 0.067 #
LVDd (mm)	3.753 ± 0.035	3.885 ± 0.113	3.883 ± 0.023	4.482 ± 0.104	4.430 ± 0.103	4.302 ± 0.156
LVDs (mm)	2.560 ± 0.047	2.642 ± 0.114	2.767 ± 0.023	3.697 ± 0.066	3.396 ± 0.091	3.588 ± 0.142
EF (%)	68.05 ± 1.86	68.26 ± 2.61	63.81 ± 0.70	43.70 ± 1.41	54.99 ± 0.78 ***	42.04 ± 0.94 ###

Supplementary Table S3. Echocardiographic parameters of GSK-3β cardiac-specific knockout (cKO) mice. GSK-3β cKO mice were fed either a fat-based low-carbohydrate (F-LC), a protein-based LC (P-LC) or control high-carbohydrate (Con) diet for 4 weeks starting at day 2 after transverse aortic constriction (TAC) or sham surgery. One-way ANOVA followed by the Tukey's post-hoc analysis. Data are mean ± s.e.m. # $p < 0.05$ and ### $p < 0.001$ compared to TAC with F-LC diet. *** $p < 0.001$ compared to TAC with control diet. HR, heart rate (HR); IVSd, interventricular septal wall thickness at end-diastole; LVDd, LV end-diastolic diameter; LVDs, LV end-systolic diameter; and EF, ejection fraction.

Supplementary methods

Mice

GSK-3β floxed mice (C57BL/6 background) were a kind gift from Dr. C.J. Phiel. Cardiomyocyte-specific deletion of GSK-3β was obtained by crossing the mice with α-myosin heavy chain promoter-driven heterozygous Cre mice (a kind gift from Dr. M.D. Schneider). Male 8 to 10-week-old animals were subjected to TAC or Sham surgery. Mice were then fed a custom diet (Fat-LC (D16102003) or Pro-LC (D16102004) diet, purchased from Research Diets) or control high-carbohydrate diet (Research Diets, D12450K) ad libitum. Daily *ad libitum* food intake was measured after an initial 2-day acclimation period on a special diet followed by a 5-day experimental period by weighing food provided and remaining every 24 hours and taking an average. Change in body weight over time was normalized as a percentage of day 1 initial starting weight for each individual animal. The sample size required was estimated to be n = 5-8 per group according to the Power analysis based upon previous studies examining the effects of pressure overload and diets on cardiac hypertrophy and hypertrophic signaling[1, 2]. We used age-matched male mice in all animal experiments. All protocols concerning the use of animals were approved by the Institutional Animal Care and Use Committee at New Jersey Medical School, Rutgers University and all procedures conformed to the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

Primary Rat Neonatal Cardiomyocytes

Primary cultures of ventricular cardiomyocytes were prepared from 1-day-old Crl:(WI)BR-Wistar rats (Envigo, Somerville) and maintained in culture. The neonatal rats were deeply anesthetized with isoflurane. The chest was opened and the heart was harvested. A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll

gradient. Cardiomyocytes were cultured in complete medium containing Dulbecco's modified Eagle's medium/F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/l bovine serum albumin (fraction V), 3 mM pyruvate, 15 mM Hepes pH 7.1, 100 μM ascorbate, 100 mg/l ampicillin, 5 mg/l linoleic acid, and 100 μM 5-bromo-2'-deoxyuridine (Sigma). Culture dishes were coated with 0.3% gelatin or 2% gelatin for immunofluorescence staining on chamber slides.

Antibodies and reagents

The following commercial antibodies were used: Rabbit monoclonal anti-mTOR (#2983), rabbit monoclonal anti-4EBP (#9644), rabbit monoclonal anti-phospho-4EBP (Thr37/46) (#2855), rabbit monoclonal anti-phospho-p70 S6 kinase (Thr389) (#9234), rabbit monoclonal anti-p70S6K (#9202), rabbit monoclonal anti-p38MAPK (#9212), rabbit monoclonal anti-phospho-p38MAPK (#4511), rabbit monoclonal anti-p44/42 MAPK (ERK1/2) (#9102), rabbit monoclonal anti-phospho-p44/42 MAPK (ERK1/2) (#4370), rabbit monoclonal anti-GSK-3α/β (#5676), rabbit monoclonal anti-phospho-GSK-3α/β (#9323), rabbit monoclonal anti-GSK-3β (#9315), rabbit monoclonal anti-phospho-GSK-3β (#5558), rabbit polyclonal anti-acetyl-lysine (#9441), and secondary antibodies (anti-mouse (#7076) or rabbit (#7074) IgG) conjugated with horseradish peroxidase (Cell Signaling Technology); mouse polyclonal anti-α-actinin (sarcomeric) (#A7811) and rabbit monoclonal anti-α-tubulin (#T6199) (Sigma-Aldrich); and rabbit polyclonal anti-BDH1 (#ab68321) and mouse polyclonal anti-OXCT1 (#ab105320) (Abcam).

Quantitative RT-PCR

Total RNA was prepared from mouse hearts and cardiomyocytes using TRIzol (Invitrogen). cDNA was generated using 300 ng total RNA and SuperScript III Reverse Transcriptase

(ThermoFisher). Using Maxima SYBR Green qPCR master mix (Fermentas), real-time RT-PCR was performed under the following conditions: 94°C for 10 minutes; 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds; final elongation at 72°C for 15 minutes. Relative mRNA expression was determined by the ΔΔ-Ct method normalized to the ribosomal RNA (18S) level. The following oligonucleotide primers were used: ANF, sense 5'-ATGGGCTCCTTCTCCATCAC-3' and antisense 5′-ATCTTCGGTACCGGAAGCTG-3′; BNP, sense 5′-AAGTCCTAGCCAGTCTCCAGA-3′
and antisense 5′-GAGCTGTCTCTGGGCCATTTC-3′; β-MHC, sense 5′-GCCAACACCAACCTGTCCAAGTTC-3′ and antisense 5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′; 18S rRNA, sense 5′-CGCGGTTCTATTTTGTTGGT-3′ and antisense 5′-AGTCGGCATCGTTTATGGTC-3′.

Immunoblotting

Cardiomyocyte lysates and heart homogenates were prepared in RIPA buffer containing protease and phosphatase inhibitors (Sigma-Aldrich) as described previously [3]. Lysates were centrifuged at 13,200 r.p.m. at 4°C for 15 minutes. Total protein lysates (10-30 μg) were incubated with SDS sample buffer (final concentration: 100 mM Tris (pH 6.8), 2% SDS, 5% glycerol, 2.5% 2-mercaptoethanol, and 0.05% bromophenol blue) at 95°C for 5 minutes. The denatured protein samples were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes by wet electrotransfer, and probed with primary antibodies.

Subcellular fractionation

Cultured neonatal rat cardiomyocytes were washed with PBS and collected with ice-cold PBS, followed by centrifugation at 600g for 5 minutes. Cardiomyocytes were then resuspended in hypotonic lysis buffer (10 mM K-HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA,

0.1 mM EDTA, 1% IGEPAL, 1% Phosphatase Inhibitor Cocktail, and 1% Protease Inhibitor Cocktail) and were incubated for 15 minutes on ice with intermittent pipetting. Whole-cell lysates were centrifuged at 1200*g* for 5 minutes. The supernatant was collected for the cytosolic fraction, and the pellets were resuspended in lysis buffer (20 mM K-HEPES, 25% Glycerol, 0.45 M NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1% Phosphatase Inhibitor Cocktail, and 1% Protease Inhibitor Cocktail) and were incubated for 15 minutes on ice with intermittent pipetting. The total homogenate was centrifuged at 13,000rpm for 10 minutes to collect the nuclear fraction. The pelleted nuclei were resuspended in lysis buffer and protein content was determined for all fractions.

Adenovirus constructs

Recombinant adenovirus vector for overexpression was constructed, propagated and titered as previously described [4]. pBHGloxΔE1,3Cre (Microbix), including the ΔE adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest into 293 cells. Replication-defective human adenovirus type 5 (devoid of E1) harboring full length wild-type Rheb cDNA (Ad-Rheb) was generated by homologous recombination in 293 cells. Adenovirus harboring beta-galactosidase (Ad-LacZ) was used as a control.

Transverse Aortic Constriction (TAC)

Mice were anesthetized with pentobarbital (60-70 mg/kg, intraperitoneal injection). The chest and neck were shaved by clipper and the skin was cleaned using betadine and 70% ethanol 3 times. Sterile ophthalmic ointment was applied to the eyes. Mice were placed in a supine position. A lack of toe pinch/tail pinch reflex was checked before making the incision. Before making the surgical incision, we subcutaneously injected a very small volume of bupivacaine along the incision line. A midline cervical incision (15-20 mm) was made to assist intubation of

the trachea and for access to the intercostal space. Mice were ventilated with a tidal volume of 0.2 ml and a respiratory rate of 110 breaths per minute. The chest was opened through the left second intercostal space. The intercostal incision was less than 0.5 cm and opened by self-designed stretchers made of 25-G needles connected to rubber bands and fixed on the surgical board by pins. With the aid of a dissecting microscope, aortic constriction was performed by ligating the transverse thoracic aorta between the innominate artery and left common carotid artery with a 26-28-gauge needle using a 7-0 prolene suture. During surgery, the depth of anesthesia was monitored periodically by checking pedal reflex. Thoracotomy incision, overlying muscle layers and skin were closed in layers using 5.0 prolene sutures, and the pneumothorax was reduced. The TAC procedure took 20-30 minutes per mouse. When recovered from anesthesia 1-2 hours after the closure of the chest, the mice were extubated and returned to their cages. Sham operation was performed without constricting the aorta. The mice were then treated with Buprenex-SR (1.0-1.2 mg/kg, SC) and monitored while being allowed to recover in a warm incubator. The animal was kept warm right before surgery and during surgery by a heating lamp, and after surgery by being kept in a warm incubator.

Echocardiography

Mice were anesthetized using 12 μl/g body weight of 2.5% avertin (Sigma-Aldrich), and echocardiography was performed using ultrasound (Vivid 7, GE Healthcare). It took around 10-20 minutes from the establishment of anesthesia to the completion of echocardiography and 1-2 hours to fully recover from anesthesia after echocardiography. A 13-MHz linear ultrasound transducer was used. Mice were subjected to 2-dimension guided M-mode measurements of LV internal diameter at the papillary muscle level from the short-axis view to measure systolic function and wall thickness, which were taken from at least three beats and averaged. LV

ejection fraction was calculated as follows: Ejection fraction = $[(LVEDD)^3 - (LVESD)^3]/(LVEDD)^3 \times 100$.

Hemodynamic measurements

Mice were anesthetized with Avertin (300 mg/kg, intraperitoneal injection) to measure arterial pressure gradients and LV end-diastolic pressures. The chest and neck were shaved by clipper and the skin was cleaned using betadine and 70% isopropyl alcohol three times. Mice were then placed in a supine position. The lack of pedal reflex was confirmed prior to making an incision. A small incision (5-10 mm) was made on the neck. Under a dissecting microscope, the common carotid artery was surgically isolated and clamped proximally and distally. A small incision (0.5-1 mm) was made in the carotid artery, and a high-fidelity micromanometer catheter (1.4 French; Millar Instruments Inc.) was inserted into the artery and advanced into the aorta to measure aortic pressure proximal to the constriction site and then into the LV cavity to measure LV pressure and its first derivatives. A separate high-fidelity micromanometer catheter was inserted via the femoral artery and advanced into the aorta to measure aortic pressure distal to the constriction simultaneously. During the procedure the depth of anesthesia was monitored by checking pedal reflex periodically to ensure the surgical plane of anesthesia.

Scientific justification for the selection of Avertin

Tribromoethanol (Avertin) is an injectable anesthetic agent. Tribromoethanol is appropriate for short procedures in mice, especially short surgical procedures such as instrumentation for hemodynamic measurements and echocardiography. Avertin was used to anesthetize mice for the following reasons: 1) Ketamine/Xylazine or Pentobarbital anesthesia suppresses cardiac function and heart rate. In general, Ketamine/ Xylazine depresses the heart rate to 200-250 beats per minute, and Pentobarbital anesthesia to ~350 beats per minute, both of which are well below

physiologic values (~650 beats per minutes in conscious mice). In comparison, Avertin has much less of a cardio-suppressive effect, with an average heart rate of around 400-450 beats per minute in mice. Due to the cardiovascular focus of our research, which includes repetitive, acute cardiovascular measurements, it is important to keep cardiac function as close to the physiological level as possible during the hemodynamic measurements; 2) Hemodynamic measurements and echocardiography do not require a long time; and 3) Avertin has few side effects[5].

Immunohistochemistry

The heart tissue was washed with PBS, fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 10-μm thickness onto a glass slide. After de-paraffinization, sections were stained with Picric acid Sirius red (PASR) for evaluation of fibrosis or wheat germ agglutinin (WGA) for evaluation of the cross-sectional area of cardiomyocytes. The outline of 100-200 myocytes was traced in each section, using ImageJ software (NIH).

Cardiomyocyte cell size

Rat neonatal cardiomyocytes were cultured on coverslips, washed with PBS twice, fixed with 3.7% paraformaldehyde for 15 minutes, and washed with PBS three times. Samples were permeabilized with PBST (0.5% Triton-X in PBS) for 15 min, and blocked in 5% BSA, 5% goat serum in PBST for 30 minutes at 37°C. Cardiomyocytes were stained with Alexa Fluor 555 phalloidin (Thermo Fisher Scientific, A34055). Samples were washed with PBS and mounted on glass slides with mounting medium containing DAPI to stain nuclei (VECTASHIELD, Vector Laboratories). Cells were observed under a fluorescence microscope. Cell size was measured in 25 - 30 cells for each condition in each experiment and the mean value was taken as a

representative of the experiment. This experiment was then performed independently five times (n = 5).

Supplementary references:

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