Supplemental Information

I. Detailed Experimental Methods

Generation and Treatment of Transgenic Animals

All animal handling and procedures were carried out in compliance with UCLA guidelines following IACUC approved protocols. The conditional αMHC-flox-MKK3bE (MKK3) mouse line [1], the tamoxifen inducible Mer-Cre-Mer mouse line (Cre, a kind gift from Dr. Molkentin, Cincinnati) [2, 3], and the MK2 null mouse line (kindly provided by Dr. Huiping Jiang, Borehinger Ingelheim Pharmaceutical Inc.) [4] were already established as described.

Experimental animals were littermates from crossbreeding between MKK3 and Cre lines either in C57BL6 widltype (WT) background or in C57BL6-MK2 null ($MK2^{-/-}$) background. The genotypes of WT, MKK3/Cre, $MK2^{-/-}$, and MKK3/Cre/$MK2^{-/-}$ were used in this experiment between 4-5 months of age. All groups of animals were treated with 20 mg/kg of tamoxifen (TX) (Sigma) in 30% ethanol/PBS injected intraperitoneally for three consecutive days. MKK3/Cre and MKK3/Cre/$MK2^{-/-}$ animals were also treated with 30% ethanol/PBS as an additional control (Veh). Following tamoxifen or vehicle injections, all mice were analyzed by echocardiography on days 0 and 7 post-induction and used for terminal experiments or sacrificed on day 8.

Echocardiography

Except where indicated, treated mice were imaged non-invasively for heart function and morphology by echocardiography on days 0 and 7 post tamoxifen treatment (injections from days 1-3). The mice were anesthetized and maintained with 2% isofluorane in 95% oxygen. A Vevo 770 (VisualSonics, Toronto, Canada) echocardiography system with a 30mHz scanhead was used to acquire the data. A parasternal short axis view was used to obtain M-mode images for analysis of fractional shortening, while a parasternal long axis view was used to obtain power doppler traces of

mitral valve filling for the calculation of the E/A ratio. The heart rate was calculated directly from the short axis view of heart contraction.

Pressure Catheterization

A subset of treated mice had their heart function measured by invasive pressure catheterization on day 8 (injections from day 1-3). The mice were anesthetized with 2g/kg urethane and maintained on a ventilator with 95% O_2, 5% CO_2. The chest cavity was opened, and an SPR-1000 micro-tip catheter pressure transducer (Millar Instruments, Houston, TX) was introduced through the apex into the center of the left ventricle. An MPCU-2000 pressure/volume conduction system (Millar Instruments) was used to control the catheter, and the data was acquired using Chart 5 (AD Instruments, Colorado Springs, CO). The resultant data was analyzed and functional parameters obtained using MPress (Millar Instruments). After the end of the experiment, the hearts were perfused with 10% formalin and used for histological analysis. None of these hearts were used for protein or mRNA analysis.

Histology

A subset of treated mice had their hearts collected for histological analysis on day 8, generally after pressure catheterization. The mouse hearts were perfused and fixed with 10% formalin prior to embedding in paraffin. For display purposes, selected hearts from each treatment group were embedded and sliced in a coronal orientation (i.e. all four chambers visible). The hearts used for analysis were embedded in a cross section orientation, and all slices were cross sections of the heart. All slices were taken from the midpoint of the ventricle. 4μm slices were deparaffinized and rehydrated prior to staining with Masson's Trichrome stain [5]. Whole mount and trichrome stained images

(40X objective) were collected using a SPOT digital camera system (Diagnostic Instruments, Sterling Heights, MI). For the purpose of calculating myocyte cross sectional area (CSA), 10 images were acquired from each heart from the epicardial region. In each image, 10 cells were quantified for their CSA using the SPOT Advanced software (Diagnostic Instruments). The resultant areas of 100 cells were all averaged to give the CSA of each heart, which counted as a sample size of 1 for statistical analysis.

ECG Telemetry

In a separate experiment, WT and MKK3/Cre mice were used for continuous ECG monitoring by a telemetry system. These mice were not imaged by echocardiography or otherwise disturbed in order to facilitate stable ECG monitoring. The heart tissue from these mice was also not used for analysis, either histological or biochemical.

The telemetry system described below was obtained from Data Sciences International (St. Paul, MN). The mice were surgically implanted with an ETA-F20 model transmitter according to the manufacturer's recommendations. The mice were then allowed to recover for 7 days before tamoxifen injection. Upon tamoxifen induction as described above, the mice were singly housed in cages on top of a RPC-1 model PhysioTel Receiver for data acquisition. The mice were left undisturbed as much as possible. The data was routed through a Data Exchange Matrix, and acquired, processed and analyzed using Dataquest A.R.T. The mice were continuously monitored for temperature, activity, and ECG waveform. The mice were allowed to die naturally, and the data analyzed for the periods prior to and just after tamoxifen induction, as well as during pathological development and at the time of death.

Cell Culture

NRVMs were isolated from P1-P3 day old Sprague-Dawley rat pups of mixed gender as described in Lu *et al.* [6]. The NRVMs were incubated in plating medium overnight after isolation, then infected with adenoviruses and incubated for an additional 48 hours in serum free DMEM medium supplemented with ITS (Invitrogen, Carlsbad, CA). In one set of experiments, a selective p38 inhibitor SB203580 (10µM, Calbiochem, San Diego, CA) was applied concurrently with the adenovirus to the cells, in serum free medium.

MEF cells were isolated from embryonic day 13 MK2 WT and KO embryos in a procedure adapted from Hogan *et al.* [7]. The resulting MEF cells were cultured in DMEM + 10% FBS. All experiments were performed between passages 3 and 6. For adenoviral transfection, MEF cells were infected with adenovirus constructs in DMEM + 2% FBS, and maintained for 48 hours before harvesting. For transient transfection, MEF cells were treated with plasmids and Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol, and maintained in serum free DMEM + ITS for 48 hours before harvest.

Adenoviral Constructs

The various adenoviral constructs were cloned, propagated and purified using the AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Final adenoviral titers were applied as multiplicities of infection (MOI) and were empirically determined for each construct.

The constitutively active (ca) and dominant negative (dn) MK2 mutants were generated by *in vitro* mutagenesis of the thr222 and thr334 activation residues to

glutamate (for ca mutant) and alanine (for dn mutant), respectively [8]. The constitutively activated caMKK3 was described previously [9].

Western Blotting and Antibodies

A subset of treated mice were sacrificed by cervical dislocation on day 8 for mRNA and protein analysis of the hearts. These mice were not used for pressure catheterization or other experiments. Hearts were removed, sectioned by chamber and snap frozen in liquid nitrogen prior to extraction of RNA or protein.

Heart total soluble protein was extracted from left ventricle pieces homogenized with a glass-glass homogenizer in a protein lysis buffer. Cell lysates were prepared in the same buffer and then sonicated. This buffer contained 20mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton-X100, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate, 1mM PMSF, 10mM NaF and a complete mini protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The lysates were also spun down in order to separate the insoluble components. Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL). 30μg of protein was separated onto 12% bis-tris polyacrylamide gels (Invitrogen) and transferred to Hybond-ECL membrane (Amersham Biosciences, Piscataway, NJ). An anti-rabbit-HRP or anti-mouse-HRP secondary antibody (Cell Signaling, Danvers, MA) with ECL detection (Thermo Scientific) was used.

The primary antibodies used were a mouse monoclonal p-p38, and polyclonal p38, p-MK2/MK2, p-ERK/ERK, GFP, p-hsp27 (all from Cell Signaling) and COX-2 (from Cayman Chemical, Ann Arbor, MI). An anti-HA antibody from Sigma and an anti α-actin antibody from Santa Cruz Biotechnology (Santa Cruz, CA) were also used. To

successfully blot for COX-2 in heart tissue, it was necessary to perform a COX-2 immunoprecipitation in these samples before immunoblotting. This assay was performed using a standard protocol (Cell Signaling) with Protein G beads and a polyclonal goat anti-COX-2 antibody (Santa Cruz). The resultant precipitate was then blotted using the COX-2 antibody from Cayman Chemical as described above.

Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Left ventricle pieces were homogenized in the TRIzol using a glass-glass homogenizer. Cell lysates were prepared directly from the culture plate. 0.5µg of the resultant total RNA was reverse transcribed into cDNA using the SuperScript II RT system (Invitrogen) with Oligo dT primers according to the manufacturer's instructions.

mRNA levels of selected genes were determined by quantitative PCR from the generated cDNA. 50µl reactions were used with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 250 nM of each primer and 1µl of cDNA. The reactions were run in a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) and the data was collected using the Bio-Rad iQ5 software (Bio-Rad). The cycler program used was an initial denaturation at 95C for 5min, 40 cycles of 45 seconds each of 95C, 60C, and 72C, another denaturation at 95C for 5min, and a final product melting curve. Data was normalized to the GAPDH mRNA levels which were run for each sample with every reaction. The threshold cycle count for each data point was subtracted from the GAPDH cycle count, and then used as in the formula x=1/2^cycle in order to facilitate data analysis. Primers were also designed to flank intronic sequences, which would reveal the

presence of genomic DNA contamination after the reaction had completed. The products were run on agarose gels to check for this contamination for every cDNA sample. The primer sequences used were as follows: GAPDH F 5'- TCC TGC ACC ACC AAC TGC TTA G -3'; GAPDH R 5'- GAT GAC CTT GCC CAC AGC CTT G -3'; COX-2 F 5'- CCA GAT GCT ATC TTT GGG GA -3'; COX-2 R 5'- CGC CTT TTG ATT AGT ACT GTA G -3'; ANF F 5'- CTG ATG GAT TTC AAG AAC CTG CT -3'; ANF R 5'- CTC TGG GCT CCA ATC CTG TC -3'; βMHC F 5'- CTC AAC TGG GAA GAG CAT CCA -3'; βMHC R 5'- CCT TCA GCA AAC TCT GGA GGC –3'; Collagen I F 5'- GAC GCC ATC AAG GTC TAC TG -3'; Collagen I R 5'- GAA TCC ATC GGT CAT GCT CT -3'; TNFα F 5'- CTC TTC AAG GGA CAA GGC TG -3'; TNFα R 5'- TGG AAG ACT CCT CCC AGG TA -3'.

Induction of COX-2 In Vivo *by LPS*

MK2 WT and KO mice were treated with LPS in order to induce COX-2 expression in the heart. LPS (Sigma) from *S. typhosa* was dissolved in sterile saline and injected intraperitoneally at a dose of 5 mg/kg. Six hours post injection, mice were sacrificed by rapid cervical dislocation. Hearts were removed and snap frozen in liquid nitrogen. COX-2 protein and mRNA levels were determined as described above.

Metabolic Labeling of COX-2 in MEF Cells

Cells were first pre-incubated for 48 hours in DMEM + 2% FBS followed by incubation for 15 minutes in methionine free DMEM with 25mM HEPES and 2% dialyzed FBS (Invitrogen). The cells were labeled with ^{35}S-methionine (0.1 mCi/ml, Perkin Elmer, Boston, MA). For the protein translation assay, the cells were labeled with the radioactive medium for a time course from 2 to 4 hours before harvesting. For the

protein stability (pulse/chase) assay, the cells were pulse labeled for 30 minutes, and then chased with medium containing 15 mg/L of non-radioactive methionine for an additional 0 to 8 hours before harvesting.

Labeled cells were harvested for protein preparation as described above, and the protein concentration quantified by a BCA assay (Thermo Scientific). For each experiment, a constant amount of protein from each lysate was subjected to immunoprecipitation for COX-2 using a standard protocol (Cell Signaling), Protein G agarose beads, and a goat anti-COX-2 polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitates were separated on an SDS-PAGE gel and an autoradiogram of the gel was recorded on film after exposure for 7 to 21 days. The signals were quantified using Scion Image (Scion Corporation, Frederick, MD) and the density data averaged for each time point and fit with either a linear (translation experiments) or negative exponential curve (degradation experiments) using Excel (Microsoft, Redmond, WA) [10]. The protein synthesis rates (K) and time constant of degradation (τ) were derived from the fitted line ($y_t = Kt+C$) or curve($y_t = C\, e^{t/\tau}$).

The total levels of metabolic labeling for each sample were also determined as an experimental control for global protein degradation. A constant amount of protein from each sample was precipitated by 20% trichloroacetic acid (TCA) and counted for radioactivity in a liquid scintillation counter. The resultant values were fitted to an exponential curve as described above to calculate the global protein degradation time constant.

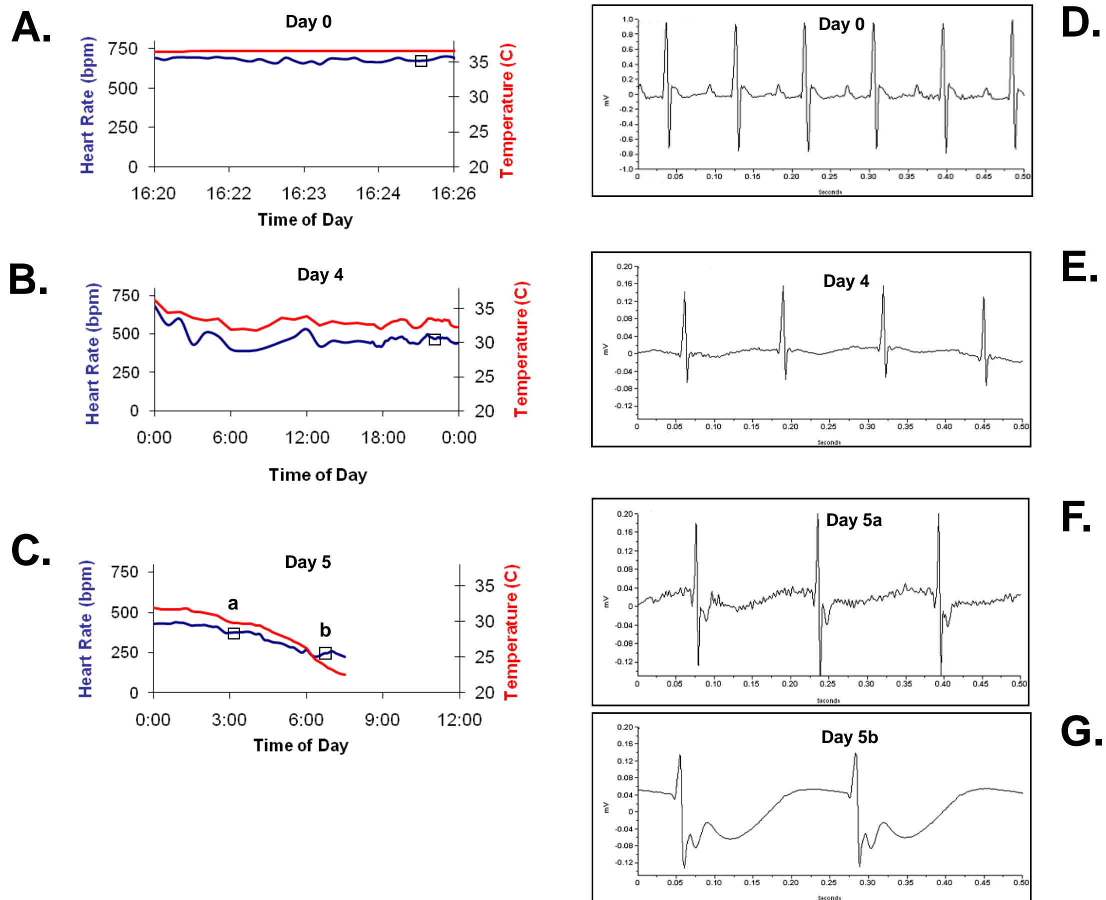
COX-2 mRNA 3'UTR Luciferase Reporter

The COX-2 mRNA 3'UTR reporter used an SV40 promoter to drive constitutive expression of a destabilized *renilla* luciferase. The full length COX-2 3'UTR followed the luciferase, and thus alterations in luciferase expression should be due to the activity of the 3'UTR. The reporter was transiently transfected as described above into WT and KO MEF cells, and the cells were incubated for 48 hours in serum free DMEM + ITS followed by treatment with either medium or medium plus 5 µg/ml of *S. typhosa* LPS (Sigma) for 6 hours. A Luciferase Kit (Promega, Madison, WI) was used to measure the *renilla* luciferase activities from protein extracts prepared from the cells according to manufacturer's instructions. A Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) was used to measure the signal with a 10 second integration time. Each sample was performed in triplicate, and the average signal was then normalized to the protein concentration of that sample. At least three sets of experimental samples were analyzed for each condition.

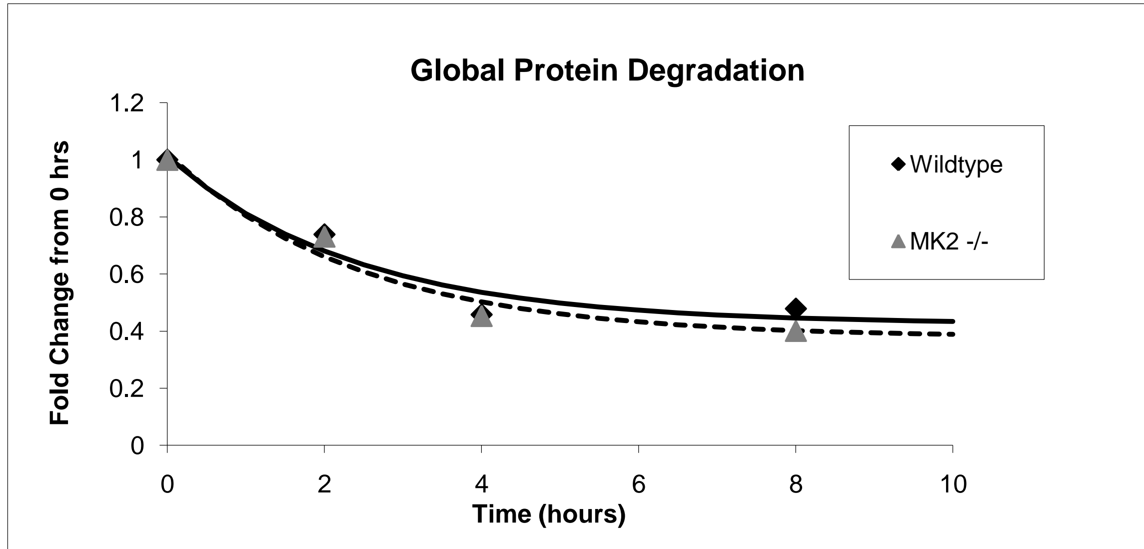
Statistical Analysis

Comparisons between more than two groups were accomplished using a factorial analysis of variance (ANOVA) with Fisher's Protected Least Significant Difference post-hoc test. Comparisons between two groups were accomplished using an unpaired two-tailed T-test. Comparisons between the survival curves were accomplished using a chi-square test. Analysis was carried out using the StatView program (Abacus Concepts, Berkeley, CA). In all cases a significant result was defined as $p<0.05$.

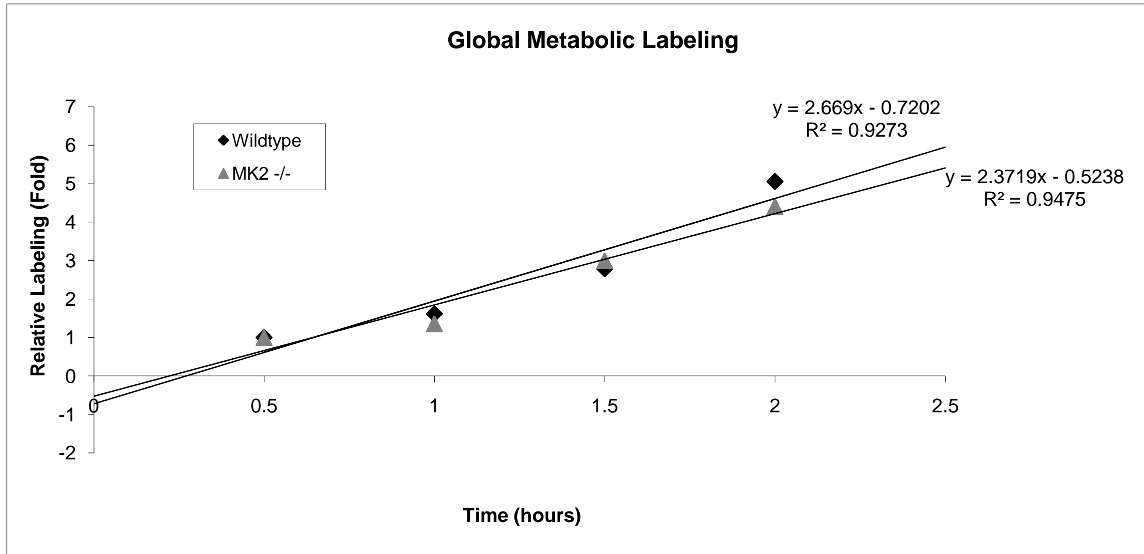
II. Supplemental Data



Online Figure I: Continuous ECG monitoring of transgenic mice. MKK3/Cre mice were implanted with a continuous ECG monitoring telemetry device, and induced with tamoxifen. Examples of heart rate and body temperature data are shown here before (Day 0) (A, D) and after tamoxifen induction at Day 4 (B, E) and Day 5 (C, F, G) with representative ECG traces illustrated for the time point indicated in the boxes (D-G).



Online Figure II: Global degradation rates in MEFs. MEFs were labeled for 30 mins. with ^{35}S-methionine, then transferred to non-radioactive medium. At the time shown the radioactivity remaining in the TCA precipitable protein for wildtype (♦ solid line) or MK2$^{-/-}$ (∇ dashed line) MEFs was measured. Degradation rates were calculated based on curve fitting as described in the Supplementary Methods.



Online Figure III: Global synthesis rates in MEFs. MK2 WT and MK2$^{-/-}$ cells were labeled with ^{35}S-methionine for the indicated time course. Total protein was harvested. At the time shown the radioactivity remaining in the TCA precipitable protein for wildtype (♦ solid line) or MK2$^{-/-}$ (∇ dashed line) MEFs was measured. Synthesis rates were calculated based on linear curve fitting as described in the Supplementary Methods.

Online Table I: Hemodynamic parameters of mice

Group	WT + TX	MKK3/Cre + TX	MK2$^{-/-}$ + TX	MKK3/Cre/MK2$^{-/-}$ +TX
HR	597.8 ± 49.5	537.7 ± 39.3	482.0 ± 86.7	582.3 ± 56.9
LVDP	84.1 ± 3.7	52.6 ± 3.6*	90.6 ± 3.1	95.3 ± 14.0 &
Pmax	87.2 ± 3.3	62.7 ± 4.9*	114.2 ± 15.2†	102.8 ± 12.5 &
Ped	4.9 ± 0.9	13.3 ± 2.8*	4.1 ±1.6	9.6 ± 3.6
dP/dT_{Max}	9307 ± 1596	6291 ± 574	6550 ± 222	9264 ± 2737
dP/dT_{Min}	-6964 ± 1126	-4182 ± 624	-6572 ± 072	-7502 ± 2015
Tau	5.52 ± 0.44	8.43 ± 1.02*	3.85 ± 0.27	8.59 ± 0.82**

Data reported as mean ± standard error. HR, heart rate (beats/min); LVDP, left ventricle developed pressure (mmHg); Pmax, maximum pressure (mmHg); Ped, end diastolic pressure (mmHg); dP/dT, rate of pressure change (mmHg/sec); Tau, time constant of relaxation. * p<0.05 vs. WT+TX, ** p<0.05 vs. MK2$^{-/-}$ + TX, † p<0.05 vs. WT + TX; &, p<0.05 vs. MKK3/Cre +TX

Online Table II: Heart and lung mass in mice

Groups	WT + TX	Cre + TX	MKK3/Cre + Veh	MKK3/Cre +TX	MK2$^{-/-}$ + TX	MKK3/Cre/ MK2$^{-/-}$ +Veh	MKK3/Cre/ MK2$^{-/-}$ +TX
LVW/BW	3.11 ±0.05	3.56 ±0.10	3.27 ±0.14	**4.52 ±0.45***	3.24 ±0.23	3.28 ±0.07	**3.82 ±0.23&**
LAW/BW	0.07 ±0.01	0.12 ±0.01	1.18 ±0.90	**2.19 ±0.71***	0.10 ±0.02	0.16 ±0.01	**0.54 ±0.15#**
LuW/BW	5.75 ±0.78	5.89 ±0.26	7.39 ±1.14	**12.25 ±2.41***	4.26 ±0.24	4.14 ±0.15	**9.47 ±1.50***

Data reported as mean ± standard error. LVW/BW, ratio of left ventricle weight over body weight (mg/g); LAW/BW, ratio of left atrial weight normalized to body weight (mg/g); LuW/BW, ratio of lung weight normalized to body weight (mg/g). * p<0.05 vs. WT + TX; &, p=0.054, vs. MKK3/Cre +TX; #, p < 0.05 vs. MKK3/Cre+TX

III. Supplementary References

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