Supplemental Materials

Ramkumar N et al. Loss of Soluble (Pro)renin Receptor Attenuates Angiotensin-II Induced Hypertension and Renal Injury

Expanded Materials and Methods

Animal Care

All animal studies were conducted with the approval of the University of Utah Animal Care and Use Committees in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Genotyping

Tail DNA was isolated and PCR was performed using the following primers: forward primer 5'-GATTTAGGATATACAGGATGCTGAGCAT-3' and reverse primer 5'-TCACCCTCAGCTTCAAGATTTCG-3' which yields a 630 bp product. DNA sequencing was performed to identify mutant sPRR mice and wild-type controls.

Histology and Immunofluorescence

Kidneys, heart and aorta were fixed overnight in 10% formaldehyde and embedded in paraffin; 4-μm sections were obtained. Sections were rehydrated with xylene and ethanol. Some sections were stained with hematoxylin and eosin per protocol by ARUP Research Histology lab at the University of Utah.

For immunofluorescence, deparaffinized kidney, heart and aorta sections were treated with 1% SDS for 10 min, blocked with 1% BSA in PBS for 1 h and incubated with primary antibody against LAMP-2 (1:50; catalog no. ABL-93, DSHB, Iowa City, IA) overnight. After 3 consecutive washes of 5 min each with PBS, sections were incubated with secondary donkey anti-mouse Alexa Fluor 488 antibody (1:100; catalog no: A-21202, Thermo Fisher) for 60 min. After three wash-rinse steps of 5 min each with PBS,

slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and were sealed with a coverslip. Tissue sections were examined and photographed with an Olympus IXplore inverted microscope.

To assess cardiac myocyte area, deparaffinized heart sections were stained with wheat germ agglutinin (WGA, Alexa Fluor 488, catalog no: W11621, Thermo Fisher) for 30-min at room temperature, followed by DAPI (catalog no: D1306, Thermo Fisher) for 5-min at room temperature. Ten 20X images were randomly acquired per section with an Olympus fluorescence camera, and cardiomyocyte cross sectional area (μm^2) was quantified using CellSens Dimension software. For all immunofluorescence experiments, controls with no antibody and secondary antibody only were performed to assess background autofluorescence and specificity respectively.

Western blot analysis

Whole kidney, heart, brain, aorta and mesenteric arteries were homogenized in ice-cold isolation buffer (50 mM Tris; 5 mM EDTA, 1% Triton, 1 mM PMSF) and complete protease inhibitors (Roche, Pleasanton, CA). Protein content was determined using the modified Lowry assay, and samples were solubilized with Laemmli loading buffer containing 0.5% lithium dodecyl sulfate. Loading control gels were initially run on 12% Bis-Tris gels, stained with Coomassie blue, and random bands were quantified by densitometry to assess equal loading. Equal amounts of protein (20 µg/lane, except for mesenteric arteries 8 µg/lane) were run on a denaturing NUPAGE 4–12% Bis-Tris minigel (Invitrogen) and were transferred to a polyvinylidene difluoride plus nylon membrane. Membranes were incubated with specific antibodies against PRR (1:1000,

cat. no. HPA003156, Sigma Aldrich, St. Louis, MO), p62 (1:1000; cat. no. ab109012, Abcam, Cambridge, MA), ERK1/2 (1:500, cat. no. 9122S, Cell Signaling, Danvers, MA), p-ERK1/2 (1:500, cat. no. 9106S, Cell Signaling) and GAPDH (1:2000; cat. no. 2118S, Cell Signaling). Secondary horseradish peroxidase-conjugated antibodies (goat anti-mouse, catalog no: ab97023 for p-ERK1/2 and goat anti-rabbit, catalog no: ab6721 for other blots, Abcam, Cambridge, MA) were used at a dilution of 1:2000. Immunoblots were visualized with the C-Digit blot scanner (LI-COR Biosciences, Lincoln, NE). Densitometry was performed with a Bio-Rad gel documentation system (Bio-Rad, Hercules, CA). To determine linearity of signal intensity, samples were run using gels loaded with ×1 and ×½ protein.

Body composition analysis

Body composition analysis (n=6/group) was performed using Bruker Minispec NMR at the Metabolic Phenotyping Core at the University of Utah in conscious control and mutant sPRR mice between 2-3 months of age.

Indirect colorimetry analysis

Energy expenditure and food intake were measured using comprehensive laboratory animal monitoring system (CLAMS) metabolic chambers (Columbus Instruments, Columbus, OH) at the Metabolic Phenotyping Core at the University of Utah. Direct measurements of oxygen consumption, carbon dioxide production, food and water intake were obtained for 48 hours (n=6/group).

Blood pressure monitoring

BP was recorded via telemetry (TA11-PAC10; Data Sciences International, St. Paul, MN). Control (n=6) and mutant sPRR mice (n=5) of 2-3 months age were anesthetized with 2% isoflurane, implanted with radio transmitters with the catheter in the carotid artery, and allowed to recover for 5 days. Automated BP and heart rate were recorded continuously with measurements taken every 10 min. Mean arterial pressure (MAP) was calculated as (1/3 × pulse pressure) + diastolic pressure. Mice were maintained on a normal Na^+ diet for 3 days. Mice were not handled during the BP recording period since even small stimuli can markedly affect BP.

Glomerular filtration rate measurement

Glomerular filtration rate (GFR) was measured in control and mutant sPRR mice on days 10-11 of the second Ang-II infusion. Mice were injected with fluorescein isothiocyanate-sinistrin (Medibeacon, Mannheim, Germany) retro-orbitally (7.5 mg/100 g body weight). The NIC-Kidney (Medibeacon) was used to detect fluorescence in the skin on the shaved back over 1 hour. GFR was calculated based on the kinetics of fluorescence decay as previously reported [22].

Echocardiography

Mice were anesthetized with 2% of isoflurane and echocardiography was performed using a Vevo 2100 system (VisualSonics, Toronto, Canada) with a 55-MHz transducer. Left ventricular dimensions were acquired from individual measurements in M-mode of the short axis view at the mid-ventricular level. Left ventricular end-diastolic

and end-systolic diameter and volume, fractional shortening, ejection fraction, systolic and diastolic left ventricular wall thickness, and left ventricular mass were analyzed with Vevo 1.6.0.6078 software. Cardiac output, stroke volume and E/A were calculated from acquired measurements.

Mesenteric artery vasoreactivity

Mice were anesthetized using 2-5% isoflurane, the left ventricle was slowly perfused with a physiological saline solution (PSS; pH 7.35-7.40), and the heart and entire mesentery were excised. After pinning the mesentery to a sylgard lined dish while immersed in iced PSS, two segments of second and third order mesenteric arteries were dissected free from adherent tissue.

One 20 μm thick tungsten wire was placed through the lumen of the mesenteric artery while the vessel was immersed in iced PSS in a sylgard lined dissecting dish that was affixed to an ice pack. The artery then was transferred to a myograph chamber containing 8 ml PSS, where the second tungsten wire was inserted through the vessel. One wire was attached to a stationary micrometer, while the second wire was fastened to a force transducer. After the vessel mounting procedure was complete, the tissue chamber was heated over 45-min from room temperature to 37°C with vessels at 0 mg tension. Tension on the artery then was increased 100 mg every 10-min by manually increasing the distance between the wires until 400 mg tension was achieved.

A series of internal circumference-active tension curves then was completed to determine the vessel diameter that evoked the greatest tension development (i.e., L_{max}) to 100 mM potassium chloride (KCl). To assess tension development, non-receptor

mediated vasocontractile responses to KCl (20-100 mM) were completed, followed by receptor-mediated vasocontractile responses to phenylephrine (PE, 10^{-8}-10^{-5} M) and Ang II (10^{-9}-10^{-5} M). A 30-min interval separated each dose-response curve, during which time the vessel bathing medium was exchanged at 10 and 20-min.

To assess endothelium-dependent vasorelaxation, the arteries first were precontracted above baseline by 65% of their maximal response to PE. When tension development to this precontraction was stable, responses to acetylcholine (10^{-8}-10^{-5} M) were assessed to determine stimulated endothelium-dependent vasorelaxation. Upon completion of this concentration response curve, the vessel bathing medium was exchanged twice immediately, and again at 5-min. To assess endothelium-independent vasorelaxation, PE-induced precontraction again was performed as described, and a sodium nitroprusside (10^{-9}-10^{-4} M) concentration-response curve was completed to assess the integrity of the vascular smooth muscle. All data were recorded continuously using an analog-to-digital interface card (Biopac Systems Inc., Santa Barbara, CA) that allowed for subsequent off-line quantitative analyses. Values from two mesenteric artery segments per mouse were averaged [23].

Since we observed blunted Ang-II induced vasoconstriction in mesenteric arteries from mutant sPRR mice, we performed additional experiments to identify potential mechanisms. In a separate group of control and mutant sPRR mice, mesenteric arteries were isolated and prepared as described. After determining L_{max} and vasocontractile responses to KCl (30 minutes later), mesenteric arteries from both groups were incubated i) N^G- Methyl-L-Arginine acetate (L-NMMA, 1 mmol/L; to inhibit nitric oxide synthase); (ii) indomethacin (10 μmol/L; to inhibit products of

cyclooxygenase metabolism); (iii) apamin (1 μmol/L; to inhibit small conductance Ca^{2+} -activated K^+ channels) + charybdotoxin (100 nmol/L; to inhibit intermediate and large conductance Ca^{2+} -activated K^+ channels); or (iv) or tempol (100 μmol/L; a superoxide dismutase mimetic). After treatment with each compound for 30-min, Ang II-induced vasocontraction was evaluated.

Quantitation of Ang-II receptor mRNA

RNA was isolated from mesenteric arteries of control and mutant sPRR mice and reverse transcription performed on 50 ng of total RNA with oligo(dt) and Superscript III reverse transcriptase, according to manufacturer's protocol (Invitrogen, Grand Island, NY). The resulting cDNA was assayed for relative expression of Ang-II Type IA, Type IB and Type II using TaqMan gene expression assay (Probe cat. Mm00616371-m1, Mm02620758-s1, Mm00431727-g1 respectively). PCR was performed for 40 cycles with denaturation at 95°C for 15 seconds and annealing and extension at 60°C for 1 minute using the StepOnePlus RT-PCR system. The Livak method ($2^{-\Delta\Delta CT}$) was used for analyses.

Blinding

All surgical procedures, vascular reactivity studies, histological analyses, GFR measurement and echocardiography were performed by individuals blinded to genotype and treatment groups.

Power calculation

Based on our previous experience, we anticipated a 20 mm Hg difference in blood pressure following Ang-II infusion between control and mutants. Assuming alpha of 0.05, power of 90% and standard deviation of 10 mm Hg, we calculated needing n=5 mice/group.

Online Table I. Blood gas analyses in control and mutant sPRR mice at baseline

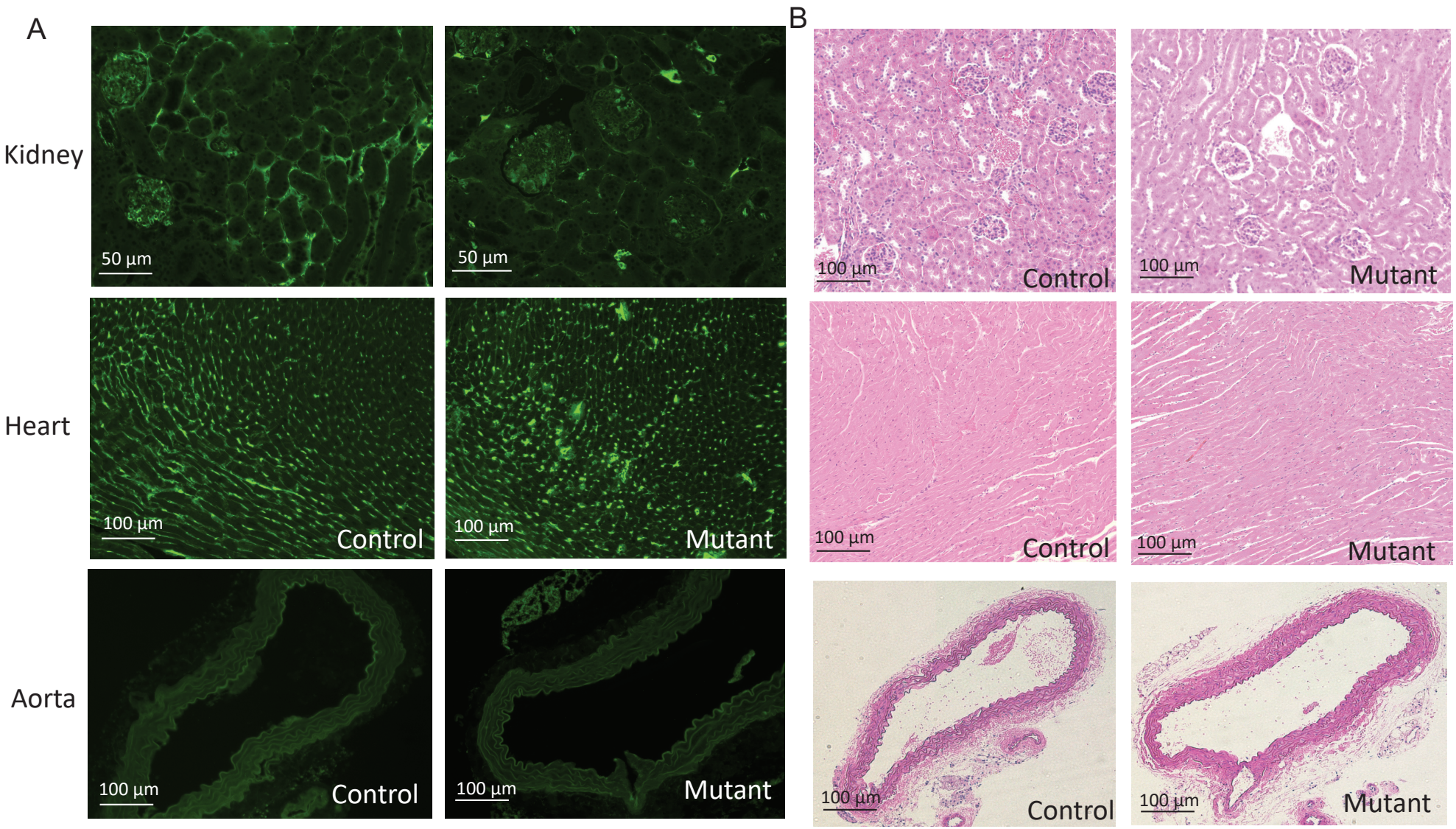
	Control (N=5)	Mutant (N=4)
pH	7.24 ± 0.02	7.21 ± 0.05
pCO_2 (mm Hg)	42 ± 2	48 ± 8
HCO_3 (mmol/L)	17.8 ± 1.4	18.5 ± 1.6
Na^+ (mmol/L)	146 ± 1	147 ± 1
K^+ (mmol/L)	4.6 ± 0.2	5.1 ± 0.2
Cl^- (mmol/L)	118 ± 1	119 ± 1
Glucose (mg/dl)	250 ± 21	217 ± 24
BUN (mg/dl)	27 ± 1	23 ± 2
Hgb (g/dl)	13.2 ± 0.3	12.4 ± 0.5
Hct (%)	38.6 ± 0.9	36.6 ± 1.6

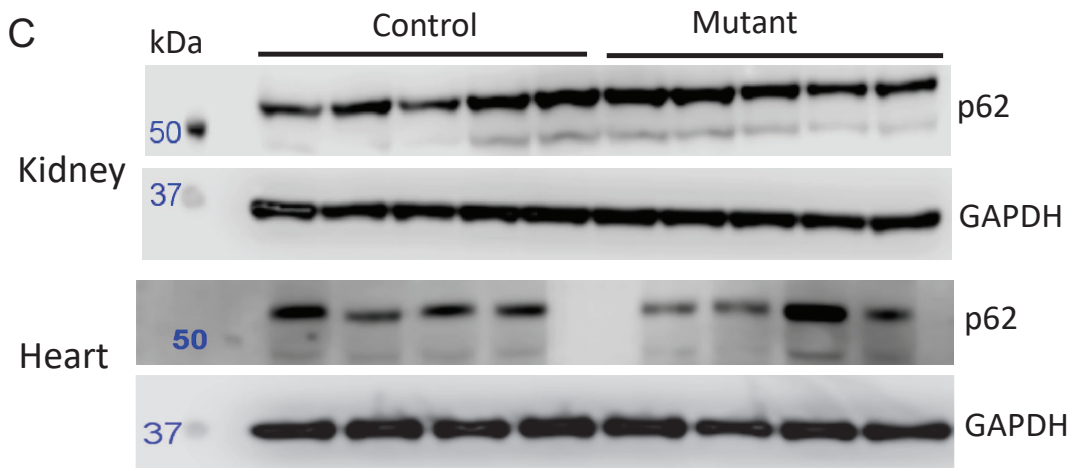
Online Table II. Tissue weights in control and mutant sPRR mice at baseline. * P <0.05 by Student's unpaired t-test

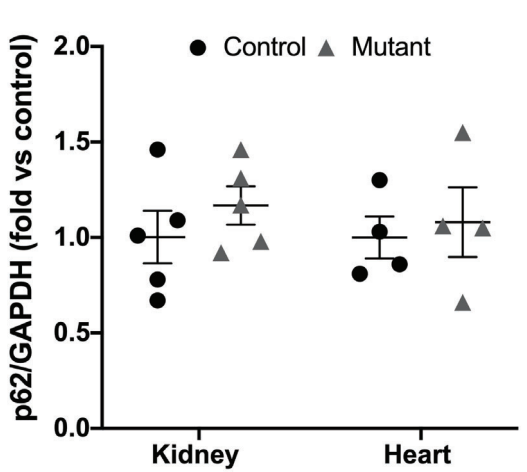
	Control (N=7)	Mutant (N=6)
Body weight (g)*	30.2 ± 2.5	25.3 ± 1.8
Kidney weight (g)*	0.16 ± 0.01	0.09 ± 0.02
Heart weight (g)	0.15 ± 0.01	0.14 ± 0.01
Liver weight (g)*	0.55 ± 0.03	0.31 ± 0.10
Epididymal fat (g)*	0.58 ± 0.13	0.43 ± 0.08

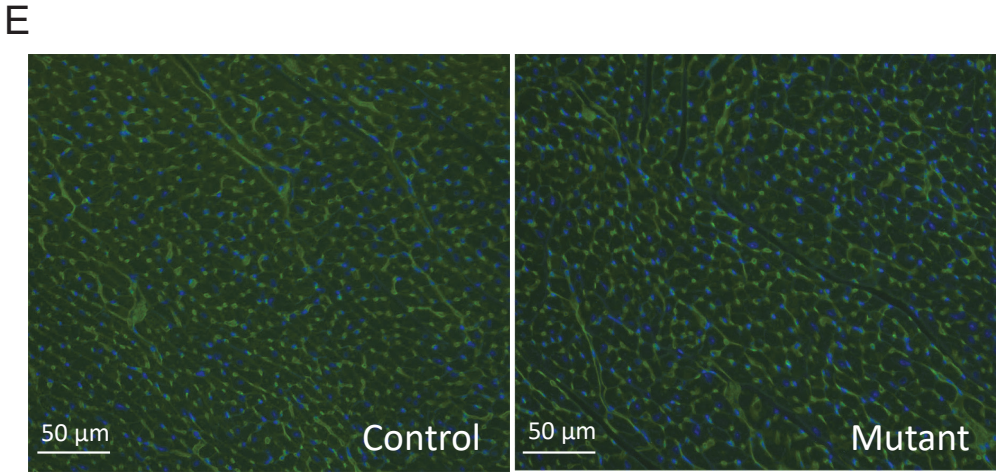
Online Table III. Vessel characteristics in control and mutant sPRR mice at basal conditions. Start width, internal diameter at 0 mg tension; end width, internal diameter at L_{max} tension; maximum KCl Δ / vessel length, maximum developed tension in response to 100 mM KCl, normalized to vessel length, achieved during the length-tension curve determination of L_{max}; tension at L_{max}, tension on vessel required to achieve L_{max}; (n=5mice/group, 2 mesenteric artery segments per mouse).

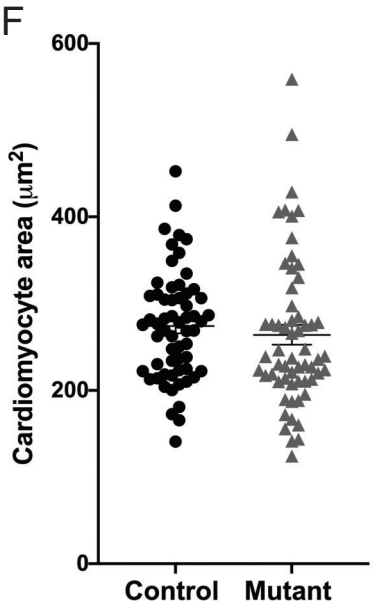
	Control (N=5)	**Mutant (N=5)**
Start width (μm)	114 ± 6	125 ± 5
End width (μm)	387 ± 11	393 ± 13
Length (μm)	1783 ± 52	1847 ± 31
Max KCl Δ / vessel length (mg/μm)	0.83 ± 0.06	0.75 ± 0.05
Tension at L_{max} (mg)	725 ± 7	735 ± 10


A
B
Kidney
Heart
Aorta
50 µm
50 µm
100 µm
Control
100 µm
Mutant
100 µm
Control
100 µm
Mutant
100 µm
Control
100 µm
Mutant
100 µm
Control
100 µm
Mutant

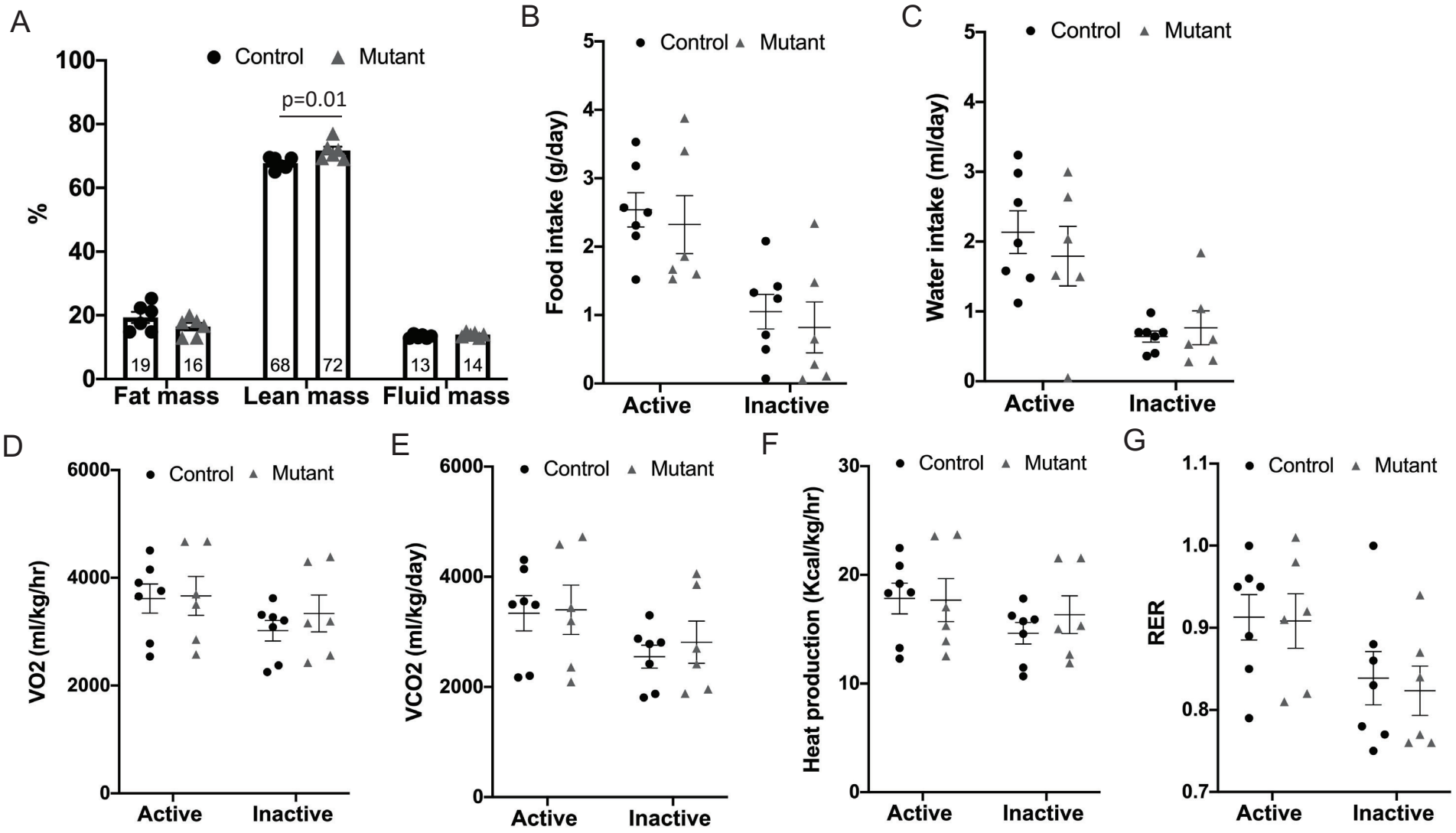

C
kDa
Control
Mutant
Kidney
50
37
p62
GAPDH
Heart
50
37
p62
GAPDH


D
Control
Mutant
p62/GAPDH (fold vs control)
2.0
1.5
1.0
0.5
0.0
Kidney
Heart

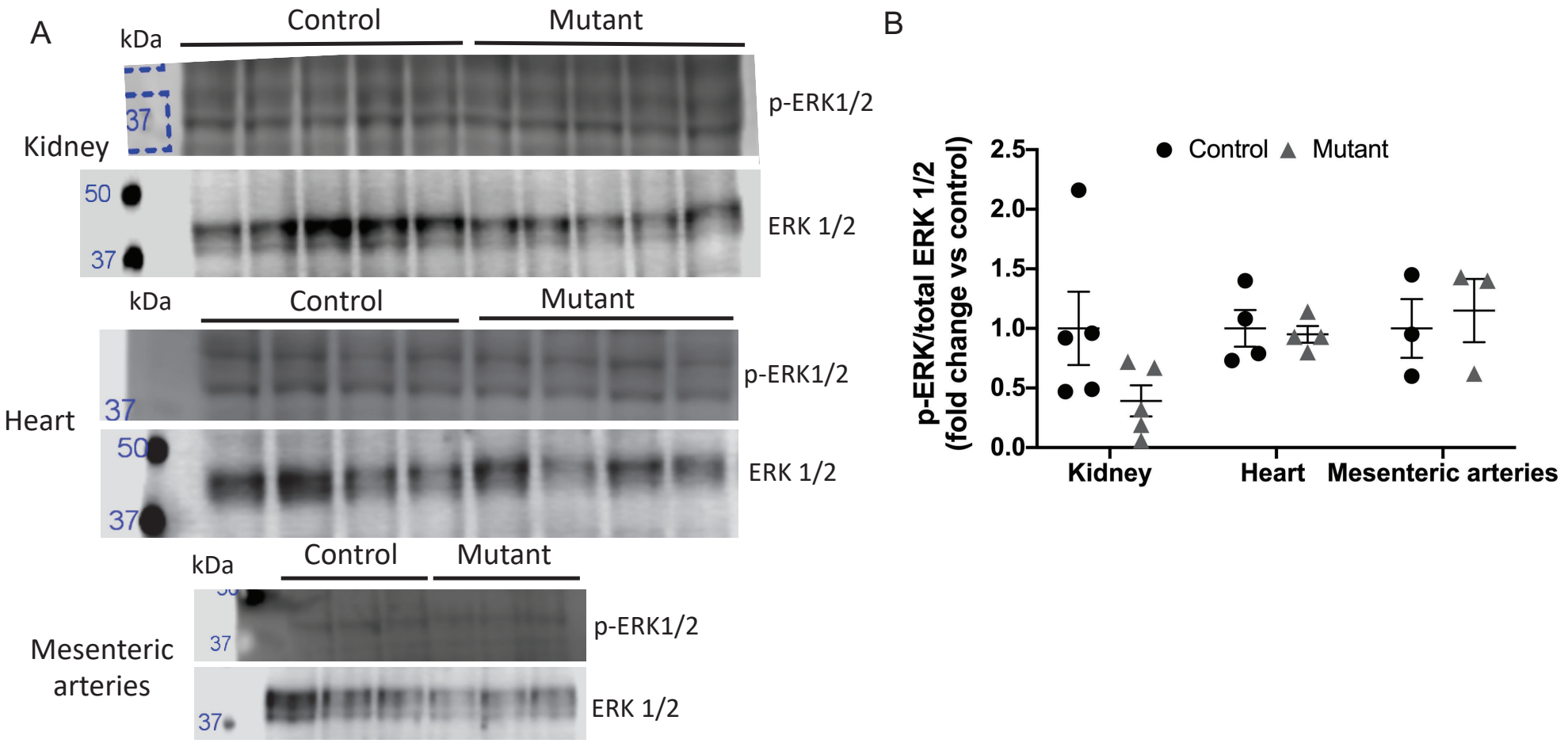

E
50 µm
Control
50 µm
Mutant


F
Cardiomyocyte area (µm2)
600
400
200
0
Control
Mutant

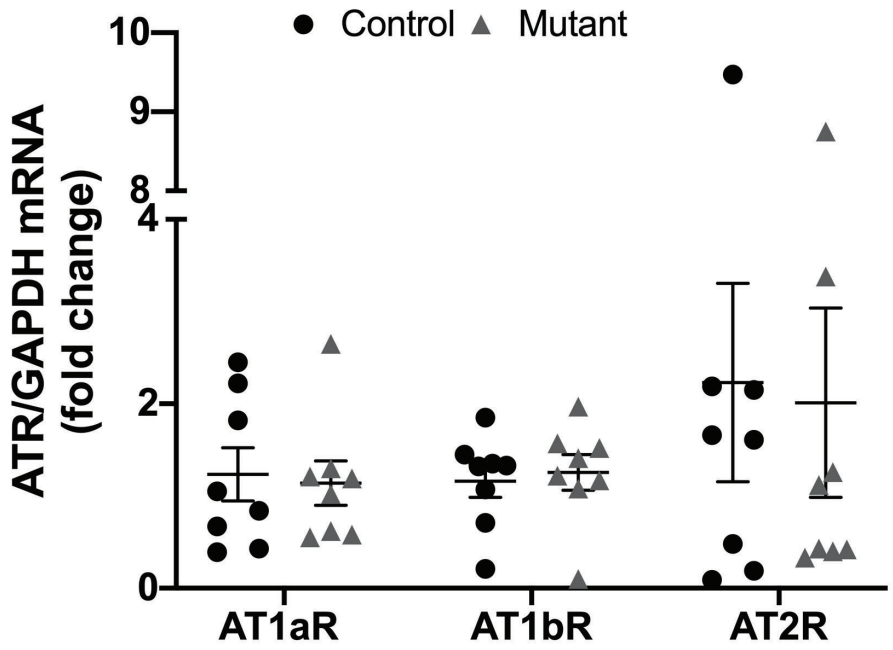
Online Figure I. Histology and autophagy markers in kidney, heart and aorta in control and mutant sPRR mice at basal conditions. A: representative images of immunofluorescent staining for LAMP-2 shows no differences between control and mutant sPRR mice (n=4/group); B: hematoxylin and eosin staining. C: western blot of p62 in whole kidney (n=5/group) and heart (n=4/group); D: densitometry of p62 in whole kidney and heart; E-F: cardiomyocyte size as assessed by WGA staining, E: representative images of WGA staining in heart sections from control and mutant sPRR mice (n=4/group); F: cross-sectional area of cardiomyocyte in control and mutant sPRR mice. Comparison between control and mutant sPRR mice was performed using Mann-Whitney non-parametric test.


A
Control
Mutant
p=0.01
%
100
80
60
40
20
0
19
16
68
72
13
14
Fat mass
Lean mass
Fluid mass
B
Control
Mutant
Food intake (g/day)
5
4
3
2
1
0
Active
Inactive
C
Control
Mutant
Water intake (ml/day)
5
4
3
2
1
0
Active
Inactive
D
Control
Mutant
VO2 (ml/kg/hr)
6000
4000
2000
0
Active
Inactive
E
Control
Mutant
VCO2 (ml/kg/day)
6000
4000
2000
0
Active
Inactive
F
Control
Mutant
Heat production (Kcal/kg/hr)
30
20
10
0
Active
Inactive
G
Control
Mutant
RER
1.1
1.0
0.9
0.8
0.7
Active
Inactive

Online Figure II. Body composition analyses and indirect colorimetry using comprehensive lab animal monitoring system (CLAMS) metabolic chambers at basal conditions in control (n=7) and mutant sPRR mice (n=6). A: body composition analyses by NMR, numbers in histogram represent %; B: food intake; C: water intake; D: oxygen consumption normalized for body weight; E: carbon dioxide production normalized for body weight; F: heat production normalized by body weight; G: respiratory exchange ratio (RER) during active (6PM-6AM) and inactive (6AM-6PM) periods. Comparison between control and mutant sPRR was performed using Student's unpaired t-test.



Online Figure III. Activation of ERK1/2 pathway in kidney, heart & mesenteric arteries in control and mutant sPRR mice. A: WB of ERK 1/2 and phosphorylated ERK1/2 in whole kidney (n=5/group), heart (n=4/group) & mesenteric arteries (n=9/group, 3 samples pooled together); B: densitometry of phosphorylated ERK/ total ERK1/2 in whole kidney, heart & mesenteric arteries. Comparison between control and mutant sPRR mice was performed using Mann-Whitney non-parametric test.



Online Figure IV. Quantitative RT-PCR of angiotensin-II receptors in mesenteric arteries from control and mutant sPRR mice at basal conditions (n=8/group). Comparison between control and mutant sPRR mice was performed using Student's unpaired t-test.