SUPPLEMENTAL MATERIAL

Supplemental Methods

Blood pressure (BP) determination by telemetry

BP was measured by radiotelemetry (HD-X11, Data Sciences International, St. Paul, Minn.)[1]. Eight-week-old BA-Bmal1-KO, BA-Agt-KO and wild type mice were anesthetized with isoflurane. The left common carotid artery was cannulated with the implant catheter, and the implant was secured in the abdominal cavity. After 1-week to allow recovery from surgery, systolic, diastolic BP, heart rate, locomotor activity and body temperature were recorded. Mice were kept on a 12/12-hour light/dark cycle. When required, Ang II infusion was performed as previously described[2].

PVAT extracts

The thoracic PVAT of 8-week-old Bmal1-KO, Agt-KO and wild type control mice were dissected and washed 3 times in ice-cold phosphate buffered saline (PBS) solution (10 mM Na_2HPO_4, 10 mM H_2PO_4, 0.9 g NaCI/100ml, pH 7.4) to remove blood in the tissue. Next, the PVAT was homogenized in ice-cold PBS (1 ml/mg of volume/weight tissue) using a glass Dounce homogenizer for 10 times on ice, and the resulting extracts were centrifuged at 5,000 rpm at 4^{o}C for 10 min to eliminate lipids and debris[3]. The liquid extracts were kept at -80^{o}C until use.

Wire myography

The recipient thoracic aortic rings (~3mm in length) were dissected from 8-week old C57BL/6 wild type, Bmal1-KO or Agt-KO mice, and the attached PVAT was cleaned in ice-cold PBS solution. To determine endothelium-dependent responses, in addition to PVAT removal, the endothelium of the aortic rings was completely removed prior to myography. Then the recipient vessel rings were mounted onto a wire myograph chamber (DMT 610M, ADInstruments) containing 10 ml physiological solution (118.99 mM NaCl, 25 mM $NaHCO_3$, 4.69 mMKCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.03 mM EDTA, and 5.5 mM glucose) at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. After equilibration and normalization procedures as described in our previous works[1], aortic contractility was determined by addition of 60 mM KCI, PVAT extracts, vasoconstrictors phenylephrine, serotonin and Ang II, or the vasodilators acetylcholine or sodium nitroprusside. For AT1 receptor inhibition, 10^{-8}-10^{-6} M losartan or valsartan were used[4]. Constriction is measured as tension and expressed in miliNewton (mN) relative to the corresponding controls as indicated in each case.

Ang II content assay

Thoracic PVAT were freshly collected from 8-week-old Bmal1-KO, Agt-KO and wild type control mice and extracts were prepared as described above. The blood was collected with EDTA to separate plasma. Ang II levels in PVAT extracts and plasma were measured by ELISA according to the manufacturer's instructions (Ang II ELISA

kit, Enzo Life Sciences, Cat# ADI-900-204).

Prostacyclin and Noradrenaline content assay

Thoracic PVAT were freshly collected from 8-week-old wild type and BA-Bmal1-KO mice. PVAT extracts were prepared as described above. The prostacyclin level was measured according to the instructions (Enzo Life Sciences, Cat# ADI-900-025). The Noradrenaline level was measured according to the instructions (IB89537, Immuno-Biological Laboratories).

Echocardiography.

Transthoracic echocardiography was performed in the supine or left lateral position of mice anesthetized with 3% isoflurane using a Visual Sonics Vevo 770 micro-imaging system. Two-dimensional, M-mode, Doppler and tissue Doppler echocardiographic images were recorded. Systolic and diastolic dimensions and wall thickness were measured in M-mode in the parasternal short axis view at the level of the papillary muscles. Fractional shortening and ejection fraction were calculated from the M-mode parasternal short axis view.

Western blot

Western blot analyses were performed as previously described[3]. Antibodies used in this study were anti-Bmal1 antibody (1:1000 dilution, Abcam ab93806), rat monoclonal to mouse anti-Agt antibody (1:1000 dilution, Life Span BioSciences, LS-C150249).

Quantitative Real-time PCR

Total RNA from mice PVAT was extracted with Trizol reagent (Cat# 15596, Life Technologies, Grand Island, NY), and the first-strand cDNA was synthesized with the SuperScript® III First-Strand Synthesis System (Cat# 18080, Life Technologies, Grand Island, NY). Next, levels of multiple mRNAs in PVAT, as indicated in the corresponding figures, were analyzed by QRT-PCR using the primers listed in the supplemental table and are expressed relative to β-actin.

Plasmids and transient transfection assays

The genomic fragment harboring a putative Bmal1-binding site (-808/-814) in the mouse Agt promoter was cloned by PCR from the mouse genomic DNA. The amplified products of 1.5 kbp upstream of the translation start site of mouse Agt gene were ligated into the pGL4-luciferase reporter vector (Promega) to generate pGL4-luc plasmids. Promoter activity was further validated by mutation of the putative Bmal1-binding site on the promoter at –808/–814 by replacing CACATG with AGACAT using the QuikChange Site-Directed Mutagenesis Kit (Stratagen). All PCR-generated constructs were verified by DNA sequencing. Luciferase activity was measured as described before[1]. In brief, HEK293 cells were transfected with the wild type or mutant pGL4-luciferase reporter plasmids and pRenilla-null as internal control (Promega) using Lipofectamine 2000 (Life Technologies) and co-transfected with either pcDNA3.1 overexpressing Bmal1 or the pcDNA3.1 vector as control. Cells were cultured for 24 hours after transfection, and cell lysates were measured using

the Dual Luciferase Reporter Assay System Kit (Promega).

Chromatin Immunoprecipitation (ChIP) assay

Mouse interscapular brown preadipocytes were isolated and immobilized by Dr. Jiandie Lin as previously described as described previously[5]. Preadipocytes were transfected with lentiviral vectors, lenti-Bmal1 (GFP-tagged, OriGENE Cat # MR209553L2V) or control lenti-GFP (OriGENECat # PS100071). After grown to confluence in differentiation medium (high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 nM insulin and 1 nM 3,3′,5-triiodo-L-thyronine), preadipocytes were induced by 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin for 48 h. Next, the cells were differentiated in differentiation medium for an additional 1 week. The cells were fixed and ChIP assays were performed using EZ-ChIP assay kit from Upstate Biotechnology (Lake Placid, NY). The size of the sonicated DNA fragments subjected to immunoprecipitation was 0.5 to 1 kbp as determined by ethidium bromide gel electrophoresis. Purified chromatin was immunoprecipitated using anti-Bmal1 monoclonal antibody. Eluted DNA fragments were purified to serve as templates for the PCR amplification. The input fraction corresponded to 10% of the chromatin solution before immunoprecipitation. Primers used to amplify the area containing this E-Box binding site (-808 to -814 bp upstream of the transcription start site) in the ChIP assay are: forward primer 5′-AGCCCATCTCAAACACCATCAAG-3′ reverse primer 5′-TGCATCGCATCCACAACTTCAA-3′, resulting in 173 bp fragment.

Supplemental Table. Primers used in the study

mRNA	Forward primer	Reverse primer
Bmal1	AGGCCCACAGTCAGATTGAAA	CCAAAGAAGCCAATTCATCAATG
Rev-rebα	CCCAACGACAACAACCTTTTG	CCCTGGCGTAGACCATTCAG
Npas2	GCTGATGTTGGAGGCATTAGATG	CATAGATGATGCTGCCGTCTGT
Cry1	TCGCCGGCTCTTCCAA	TCAAGACACTGAAGCAAAAATCG
Cry2	CCCACGGCCCATCGT	TGCTTCATTCGTTCAATGTTGAG
Per1	TCGAAACCAGGACACCTTCTCT	GGGCACCCCGAAACACA
Per2	GCTCGCCATCCACAAGAAG	GCGGAATCGAATGGGAGAATA
Per3	GGCTGCTTTGATCCTGAATTCT	GAACGCCCTCACGTCTTGAG
Dbp	CACCGTGGAGGTGCTAATGA	GCTTGACAGGGCGAGATCA
E4bp4	GCTCAAGAGATTCATAGCCACACA	GCTCGGTCAGCAGCCATTT
Clock	CTGTATGGGGTGACTTGGGGTTG	CTTGGGGAATGGTCTTGGTGCTC
Agt	GCGGAGGCAAATCTGAACAACAT	GAAGGGGCTGCTCAGGGTCACAT
Ace	CCACCAGGGCCCACTACACC	GACTTCGCCATTCCGCTGATT
Ucp1	AAAAACAGAAGGATTGCCGAAACT	TAAGCATTGTAGGTCCCCGTGTAG
Cidea	CTGTCGCCAAGGTCGGGTCAAG	CGAAAAGGGCGAGCTGGATGTAT
Elovl3	GGGCCTCAAGCAAACCGTGTG	GTTTTTCAGCCTTCATAGTGTAGT
Lpn	ACATACCGCATTTCAGGGCA	CCCAGGTATCCCGTGTCAAC
Adipoq	CATTCCGGGACTCTACTACTTCTC	GTCCCCATCCCCATACACCT
Rarres2	AGGTGAAGCCATGAAGTGCT	AACTGCACAGGTGGGTGTTT

Supplemental Figures and Figure Legends

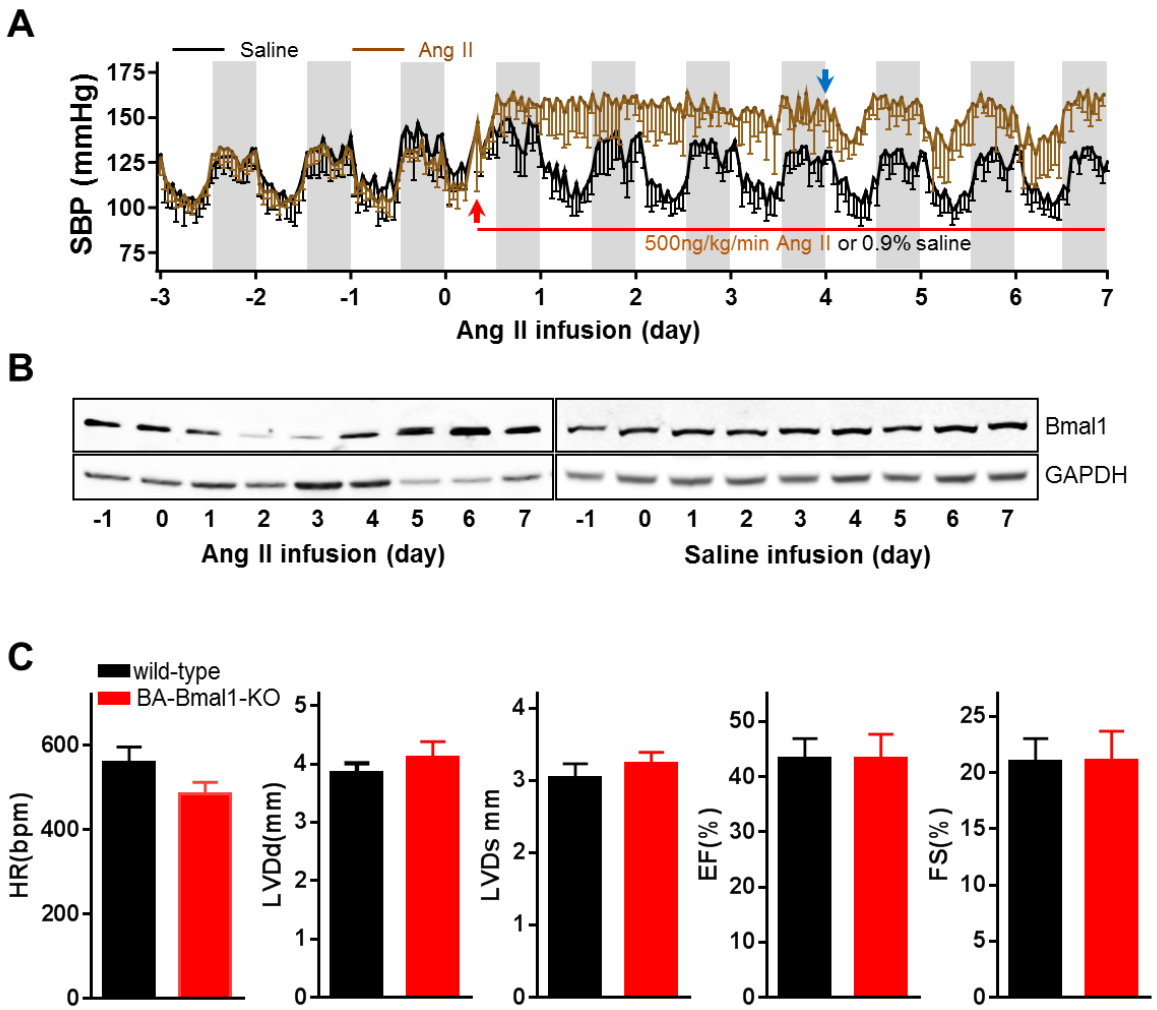

Figure I. Ang II represses Bmal1 expression in PVAT. (A), Systolic blood pressure of 10-week old C57BL/6J mice was measure by radiotelemetry. The blood pressure during day (-3) to day 0 shows normal circadian rhythmicity. Continuous infusion of Ang II by subcutaneous Alzet minipump (day 0 to day 7, red arrow indicates starting to infusion of Ang II or saline) acutely, albeit transiently, abolishes circadian rhythmicity of blood pressure with progressive restoration after day 4 (indicated as blue arrow) when compared to 0.9% saline infusion as control. Gray shade represents lights-off period from 18:00 to 6:00. Data are shown as mean ± S.D., n=6.

(B), Representative Western blot showing Bmal1 levels in PVAT of mice in (A) when the PVAT was harvested at 10:00AM. **(C)**, **Cardiac function in BA-Bmal1-KO mice.** The left ventricular end diastolic and systolic diameters (LVDd and LVDs, respectively), ejection fraction (EF) and fractional shortening (FS) were measured in wild type and BA-Bmal1 KO mice during lights-on (resting) phase using echocardiography as described in the methods section. Data shown as mean ± SD, n=5/group.

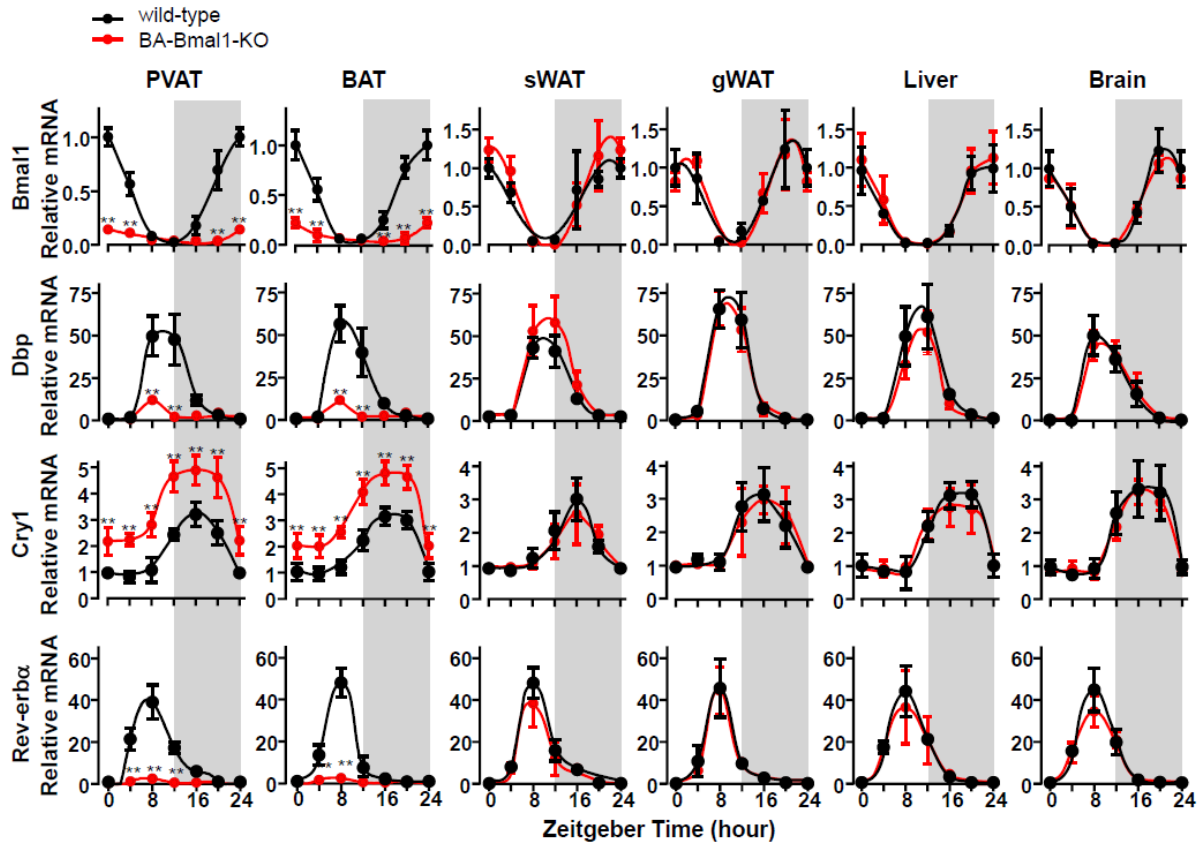

Figure II. Specific loss of circadian feedback loops in brown adipose tissues of BA-Bmal1-KO mice. PVAT, BAT, sWAT, gWAT, liver and brain of wildtype and BA-Bmal1-KO mice were collected every 4 hours. The mRNA levels of aryl hydrocarbon receptor nuclear translocator-like protein 1 (Bmal1), D-Box binding PAR BZIP transcription factor (Dbp), Cryptochrome circadian clock 1 (Cry1) and nuclear receptor subfamily 1 group d member 1 (Rev-erbα) were measured by QRT-PCR relative to β-actin. Gray shade represents lights-off period from 18:00 to 6:00. Data shown as mean ± SD, n=3/group. ***p*<0.01 *vs* wild-type.

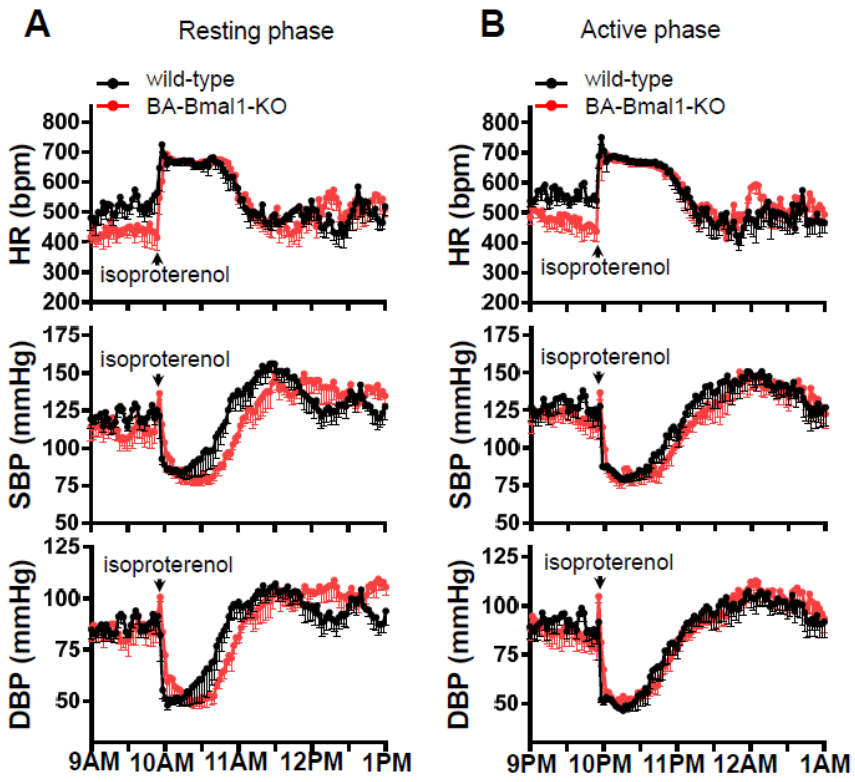

Figure III. Response of BA-Bmal1-KO mice tosystemic isoproterenol injection.

Heart rate (HR), systolic(SBP) and diastolic (DBP) blood pressure of wild type and BA-Bmal1-KO mice were measured by radiotelemetry when the temperature of the environmental chamber was set at 22^{o}C. The mice received 10mg/kg isoproterenol by i.p. injection at ~10AM, resting phase **(A)** or 10PM, active phase **(B)**. Data are shown as mean ± SD, n=5/group.

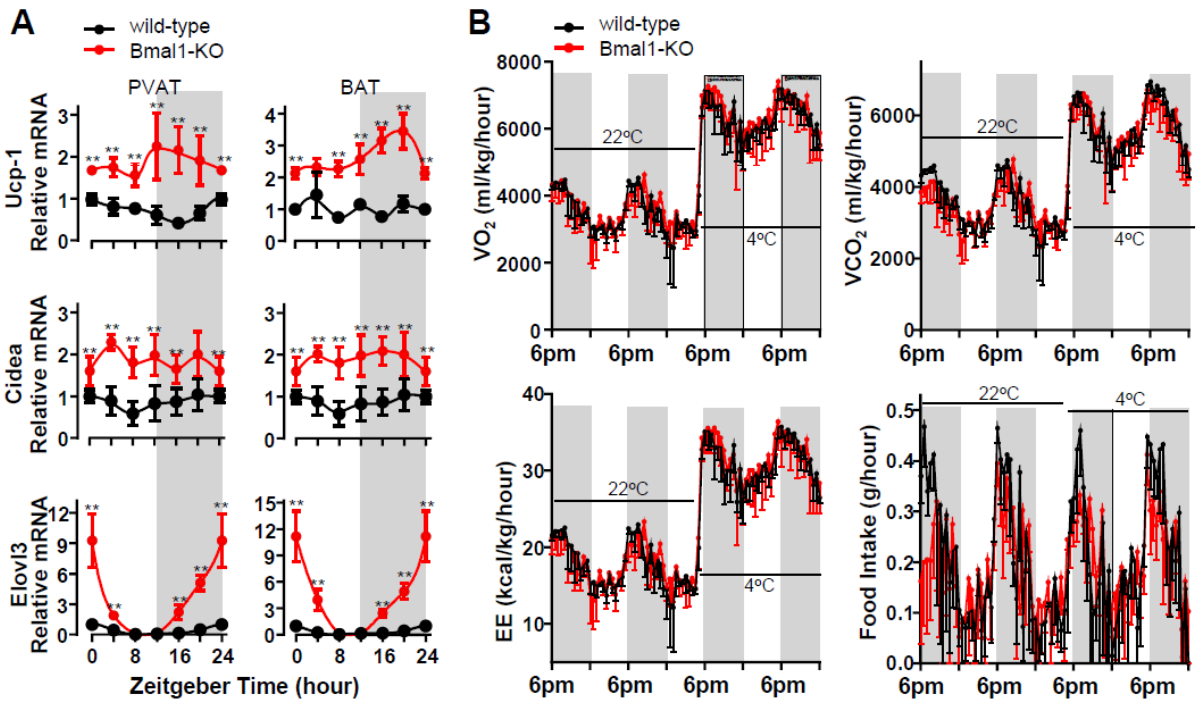

Figure IV. Thermogenic genes and metabolism. (A), mRNA levels of thermogenesis-related genes: uncoupling protein 1 (Ucp-1), cell death-inducing DFFA-like effector A (Cidea), ELOVL fatty acid elongase 3 (Elovl3) in PVAT and BAT of wildtype andBA-Bmal1-KO mice were measured by QRT-PCR relative to β-actin. Data shown as mean ± SD, n=3/group. ***p*<0.01 *vs* wild-type. **(B)**, Wild-type and BA-Bmal1-Kcice were single-housed in metabolic cages. The oxygen consumption (VO_2), carbon dioxide (VCO_2) production, energy expenditure (EE) and food intake were recorded when the chamber temperatures were set at 22°C or 4°C. Data shown as mean ± SD, n=4. Gray shade represents lights-off period from 18:00 to 6:00.

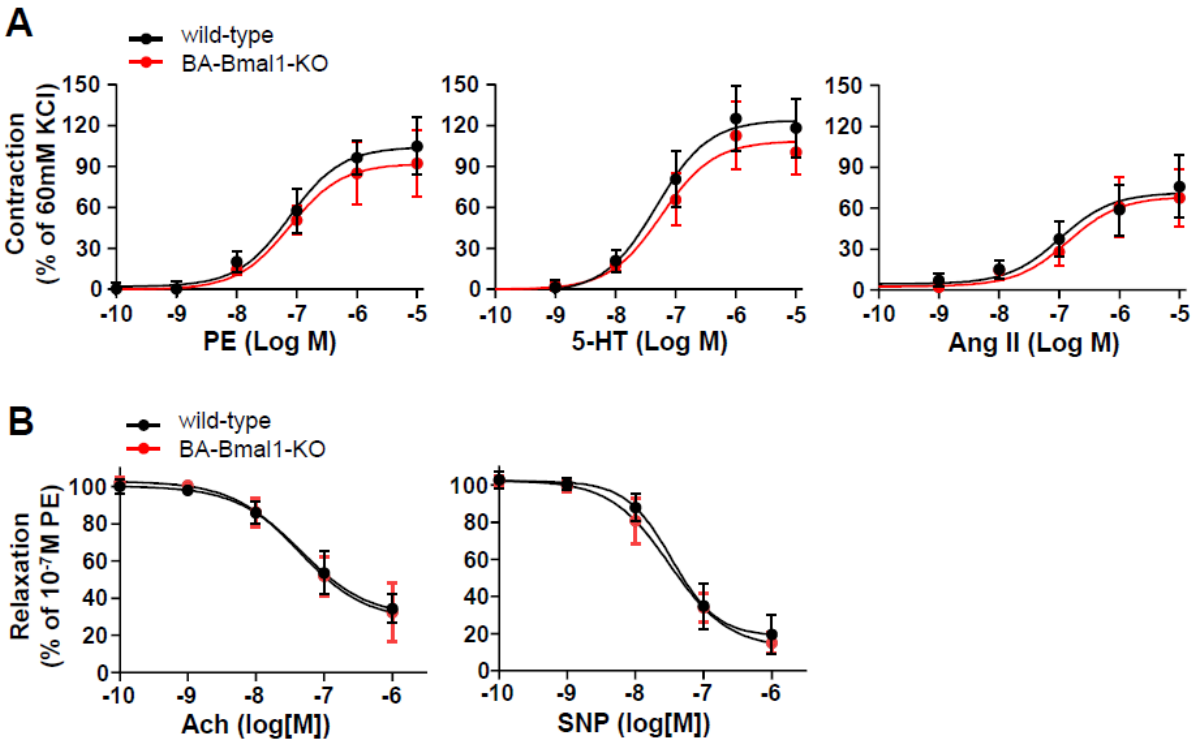

Figure V. Vasoactivity of vessel rings of BA-Bmal1-KO mice. (A), Dose-dependent contraction curves of thoracic aorta rings from wild type and BA-Bmal1-KO mice in response to phenylephrine (PE), serotonin (5-HT) and Angiotensin II (Ang II). Results are shown as mean ± SD, n=10 in each group. **(B)**, Endothelium-dependent relaxation induced by acetylcholine (Ach) in aortic rings with intact endothelium, and endothelium-independent relaxation induced by sodium nitroprusside (SNP) in aortic rings with the endothelium removed. Results are shown as mean ± SD, n=10 in each group. The thoracic aorta rings used for the above experiments were harvested during the lights-on phase and had the associated PVAT removed, as indicated in the methods section.

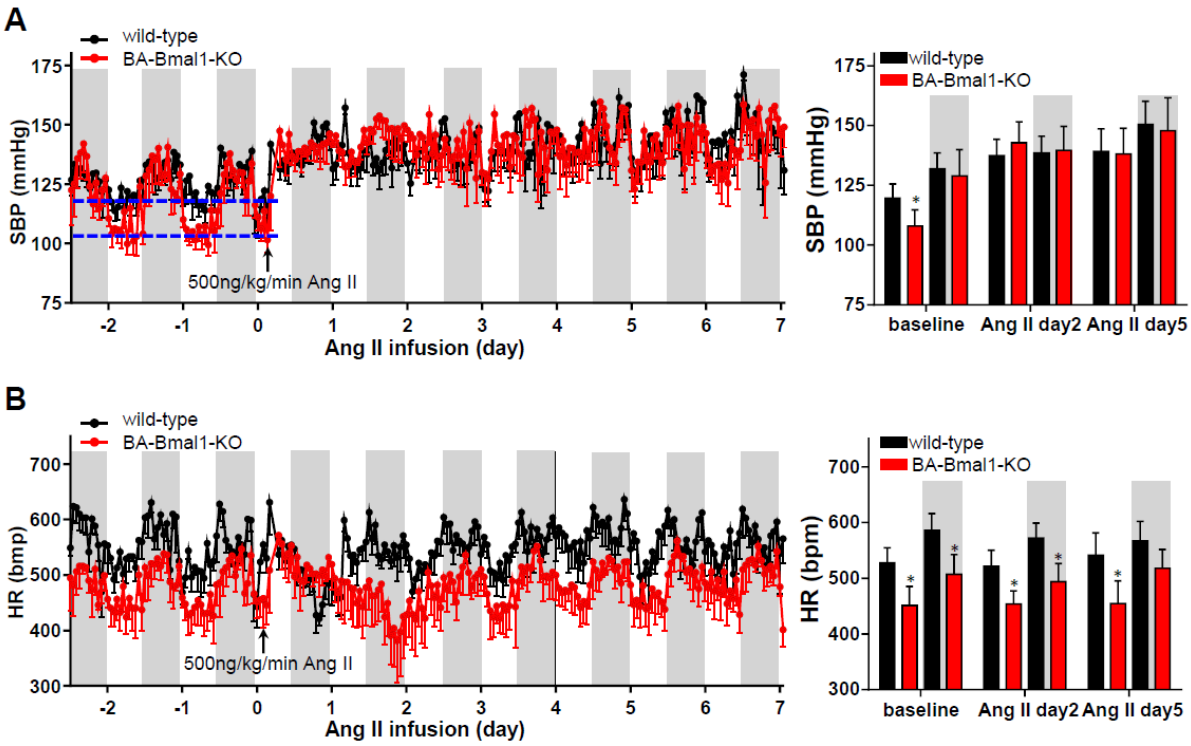

Figure VI. Systolic blood pressure and heart rate inBA-Bmal1-KO mice upon Ang II infusion. SBP **(A)** and heart rate **(B)** were measured by radiotelemetry. Day 0 represents the start day of Ang II infusion (500ng/kg/min). Blue dashed lines indicate the hypotensive phenotype in the resting phase of the BA-Bmal1-KO animals before Ang II infusion. Data are shown as mean ± SEM, n=6. *p<0.05 *vs* wild-type. Gray shade represents lights-off period from 18:00 to 6:00.

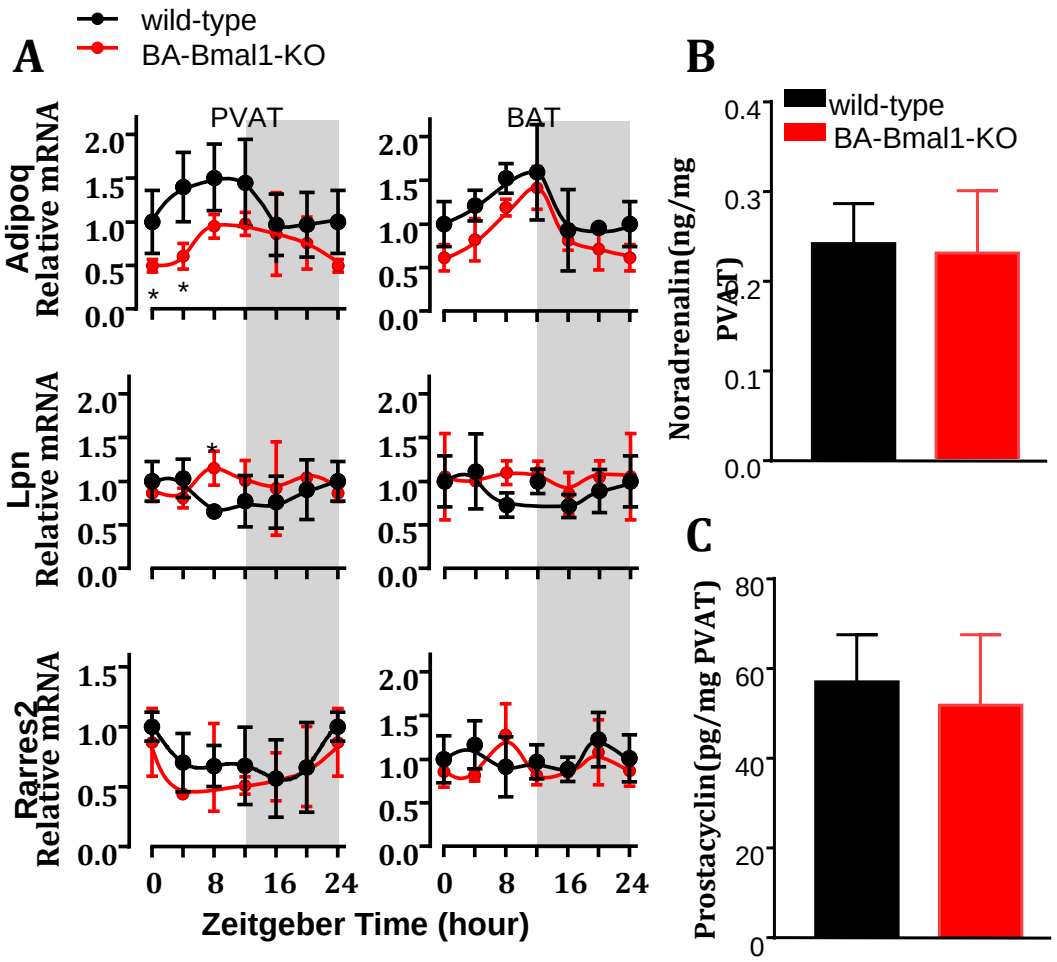

Figure VII. PVAT-derived factors are unlikely to contribute to the hypotensive phenotype.(A), mRNA levels of the anti-contractile factor adiponectin (Adipoq), contractile factors leptin (Lpn) and chemerin (Rarres2) in PVAT and BAT of wild type and BA-Bmal1-KO mice were measured by QRT-PCR relative to β-actin. Data shown as mean ± SD, n=3/group. *p<0.05 *vs* wild-type. Gray shade represents lights-off period from 18:00 to 6:00. **(B)**, adrenalin and **(C)**, prostacyclin levels in PVAT of wild-type and BA-Bmal1-KO mice. Tissues were harvested at 10AM. Data shown as mean ± SD, n=6/group.

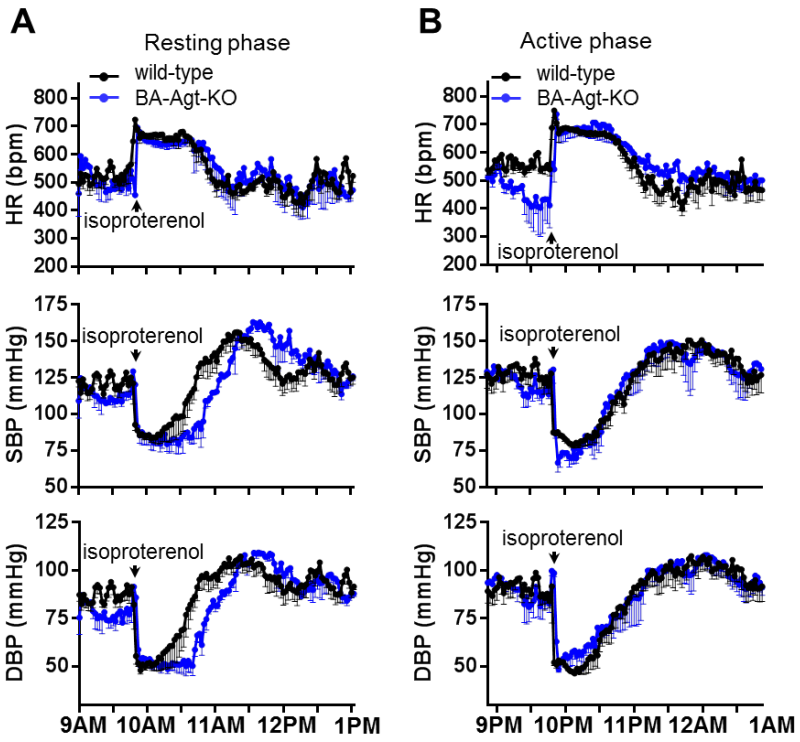

Figure VIII. Response of BA-Agt-KO mice to systemic isoproterenol injection.

Heart rate (HR), SBP and DBP of wild-type and BA-Agt-KO mice were measured by radiotelemetry when the temperature of the environmental chamber was set at 22^{o}C. The mice received 10mg/kg isoproterenol by i.p. injection at ~10AM, resting phase **(A)** or 10PM, active phase **(B)**. Data are shown as mean±SD, n=5/group.

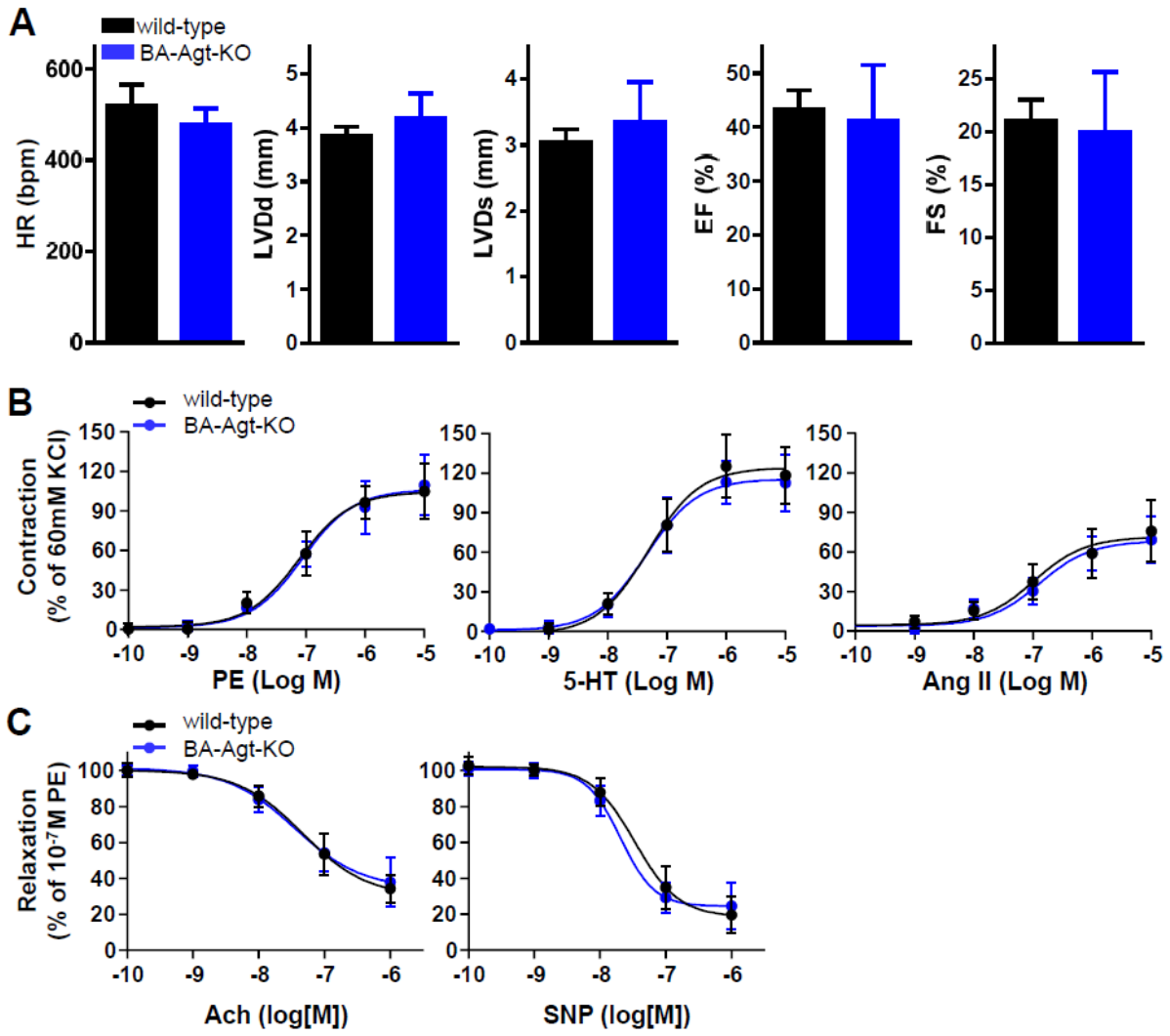

Figure IX. Cardiac function and vasoactivity of aortic rings of BA-Agt-KO mice. **(A)**, The left ventricular end diastolic and systolic diameters (LVDd and LVDs, respectively), ejection fraction (EF) and fractional shortening (FS) were measured in wild-type and BA-Agt-KO mice during lights-on (resting) phase using echocardiography as described in the methods section. Data shown as mean±SD, n=5/group.**(B)**, Dose-dependent contraction curves of thoracic aorta rings from wild-type and BA-Agt-KO mice induced by phenylephrine (PE), serotonin (5-HT) and Angiotensin II (Ang II). Results are shown as mean±SD, n=10/group. **(C)**, Endothelium-dependent relaxation induced by acetylcholine (Ach) in aortic rings with

intact endothelium, or endothelium-independent relaxation induced by sodium nitroprusside (SNP) in aortic rings with the endothelium removed. Results are shown as mean±SD, n=10/group.

References

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