SUPPLEMENTAL MATERIAL

THE CIRCADIAN PROTEIN PER1 CONTRIBUTES TO BP CONTROL AND COORDINATELY REGULATES RENAL SODIUM TRANSPORT GENES

Running title: Per1, renal gene expression and BP

Lisa R. Stow, Ph.D.[1,†], Jacob Richards[1,2], Kit-Yan Cheng[2], Lauren A. Jeffers[1], I. Jeanette Lynch[2,3], Megan M. Greenlee, Ph.D.[2,†], Brian D. Cain, Ph.D.[1], Charles S. Wingo, M.D.[2,3] and Michelle L. Gumz, Ph.D.[1,2,3]*

[1]Department of Biochemistry and Molecular Biology, [2]Department of Medicine, Division of Nephrology, Hypertension and Renal Transplantation, University of Florida, Gainesville, FL. [3]NF/SG Veterans Health Administration, Gainesville, FL.

[†]Current affiliation: SABiosciences, Frederick, MD

*To whom correspondence should be addressed

Michelle L. Gumz 1600 SW Archer Road, Box 100224, Gainesville, FL 32610

Michelle.Gumz@medicine.ufl.edu 352-273-6887 fax: 352-392-5465

Supplemental Material

Methods

Cell culture. Cells were maintained in DMEM/F12 plus 10% FBS (Invitrogen) and 50 µg/ml gentamicin. The mpkCCD$_{c14}$ cells were a kind gift of Dr. Alain Vandewalle [1] and mIMCD-3 cells were purchased from American Type Culture Collection.
RNA Silencing. RNA silencing experiments were performed as described[2]. Cells used for siRNA experiments were seeded at a density of 75,000 cells per cm^2 on 6-well Transwell plates (Corning) and transfected for 24 h with 66 nM siRNA (non-target 2 or Per1-8, Dharmacon) in 1.5 µl of DharmaFect 4. At the time of transfection cells were switched to phenol-red free DMEM/F12 plus 10% charcoal dextran stripped FBS.
Real time PCR. Quantitative real time PCR experiments (QPCR), were performed as previously described[2]. Total RNA was isolated from cells using TRIzol® Reagent (Invitrogen), treated with DNase I (Ambion) to eliminate genomic DNA, and reverse transcribed using oligo dT, random hexamers and Superscript™ III reverse transcriptase (Invitrogen). Resulting cDNAs (20 ng) were used as templates in duplicate QPCR reactions (Applied Biosystems). Cycle threshold (C_T) values were normalized against β-actin (*actb*) and relative quantification was performed using the $\Delta\Delta C_T$ method[3]. All QPCR experiments were performed with TaqMan® primer/probe sets that have guaranteed 100% PCR efficiency over six logarithms of template[4].
Western blot. Western blot analysis was performed as described[5] using antibodies against caveolin-1 (Santa Cruz), αENaC (kind gift of Dr. Carolyn Ecelbarger, Georgetown University), and Per1 (ThermoFisher Scientific).
Membrane protein preparation. Membrane proteins were collected using differential centrifugation. Briefly, mpkCCD$_{c14}$ cells were collected by scraping in ice cold PBS and centrifuging at 3000 x g for 10 minutes. Cells were resuspended in sucrose buffer A (10 mM, Tris 1 mM EDTA, 50 mM sucrose) and homogenized 25 strokes. An equal volume of sucrose buffer B (10 mM Tris, 1 mM EDTA, 250 mM sucrose) was added, followed by an additional 25 strokes homogenization. Nuclei were pelleted and discarded after 10 minutes centrifugation at 1000 x g. Organelles were pelleted and discarded following 20 minutes of centrifugation at 10,000 x g. Supernatants were centrifuged at 100,000 x g for 16 hr. Membrane protein pellets were resuspended in 75 µl of sucrose buffer B and phosphatase and protease inhibitors. Protein concentrations were quantified by BCA assay.
DNA affinity purification assays (DAPA). Nuclear extracts were obtained from mpkCCD$_{c14}$ cells using the NE-PER kit (Pierce) according to the manufacturer's instruction and subjected to DNA-affinity purification analysis (DAPA) as described[5]. Double stranded DNA probes were biotinylated on each 5' end (Sigma Genosys) and were homologous to E-box 2: 5'-AGACTTGGTGGAAGGGGTGGTGGTGGAAAAGT or E-box 1: 5'- GGATGTACCTGACAAAACCACATTGTTGTTGTTATC in the *Edn1* promoter (see Figure 5B). E-box 2 mutant sequence was 5' AGACTTGGTGGAGCTCTCTCTGTGGAAAAGT. Probes were immobilized on 50 µl of streptavidin coated agarose beads and incubated with 175 µg of nuclear extract in the presence of phosphatase and protease inhibitors (Pierce) for 1 h at room temperature with end-over-end rotation. Beads were pelleted. Pelleted beads were washed four times with ice-cold PBS plus phosphatase and protease inhibitors. After the final wash,

all liquid was aspirated from the beads with flat-headed gel loading tips. The beads were resuspended in 50 µl of 2x lithium dodecyl sulfate sample buffer (Bio-Rad) plus β-mercaptoethanol. Samples were boiled for 5 min and loaded onto a 7.5% Tris-HCl SDS-PAGE Ready Gel (Bio-Rad) for electrophoresis. The presence of Per1 in the DAPA complexes was evaluated using Western blot analysis as described above. Equal loading was controlled for by BCA assay and staining DAPA blots with Ponceau S (data not shown).

ET-1 Protein. For cell culture samples, ELISA was performed on media collected from the apical and basolateral sides of cell monolayers. One milliliter of media was dried under vacuum to be used in the assay. For animal tissues, cytoplasmic extracts were isolated from renal cortex or inner medulla using the NE-PER kit (Pierce) according to the manufacturer's instructions. Immunoreactive ET-1 peptide was detected by chemiluminescent ELISA (R&D Systems) and normalized to total protein content as determined by BCA protein assays (Pierce).

BP Measurements in Mice. Age-matched, male WT (129/sv) or *Per1* KO mice were implanted with telemetry transmitters (Data Sciences International (DSI)), enabling 24 hr measurement of BP and other parameters in conscious, unrestrained animals. Mice were maintained on normal lab chow and kept under normal light:dark conditions. Telemetry recordings were made for 2 minutes, every 10 minutes over the course of 72 hr. MAPs were averaged over each day or night period. Data were collected and analyzed using DSI software.

References for Supplemental Materials

1. Bens M, Vallet V, Cluzeaud F, Pascual-Letallec L, Kahn A, Rafestin-Oblin ME, Rossier BC, Vandewalle A. Corticosteroid-dependent sodium transport in a novel immortalized mouse collecting duct principal cell line. *J Am Soc Nephrol*. 1999;10:923-934.
2. Gumz ML, Stow LR, Lynch IJ, Greenlee MM, Rudin A, Cain BD, Weaver DR, Wingo CS. The circadian clock protein period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest*. 2009;119:2423-2434.
3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods*. 2001;25:402-408.
4. Applied Biosystems. Amplification efficiency of taqman gene expression assays. 2006
5. Gumz ML, Cheng KY, Lynch IJ, Stow LR, Greenlee MM, Cain BD, Wingo CS. Regulation of alphaenac expression by the circadian clock protein period 1 in mpkccd(c14) cells. *Biochim Biophys Acta*. 2010;1799:622-629.
6. Farre D, Roset R, Huerta M, Adsuara JE, Rosello L, Alba MM, Messeguer X. Identification of patterns in biological sequences at the alggen server: Promo and malgen. *Nucleic Acids Res*. 2003;31:3651-3653.
7. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. Promo: Detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics*. 2002;18:333-334.

Table S1. Summary of telemetry data

Variable	WT	*Per1* KO	P value
Signal	28.802	28.795	0.987
Activity	3.99	3.38	0.493
Pulse	13.619	14.485	0.799
Heart Rate	519.118	504.36	0.65

WT: wild type, KO: knockout

Supplemental Results

Supplemental Figure 1.

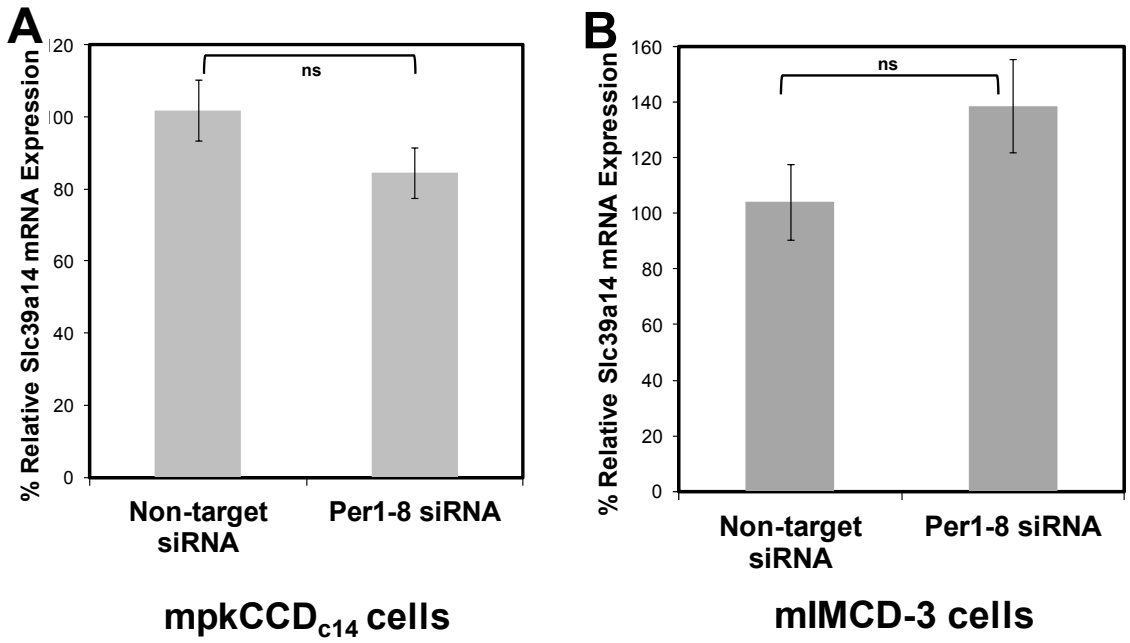

Figure S1. Effect of Per1 knockdown on expression of the Zinc transporter Slc39a14. mpkCCD$_{c14}$ or mIMCD-3 cells were transfected with a non- target siRNA or Per1 specific siRNA. Under these conditions, Per1 mRNA levels were reduced by about 90%. QPCR was used to measure changes in gene expression following Per1 knockdown for Slc39a14 in A. mpkCCD$_{c14}$ cells or B. mIMCD-3 cells.

Supplemental Figure 2

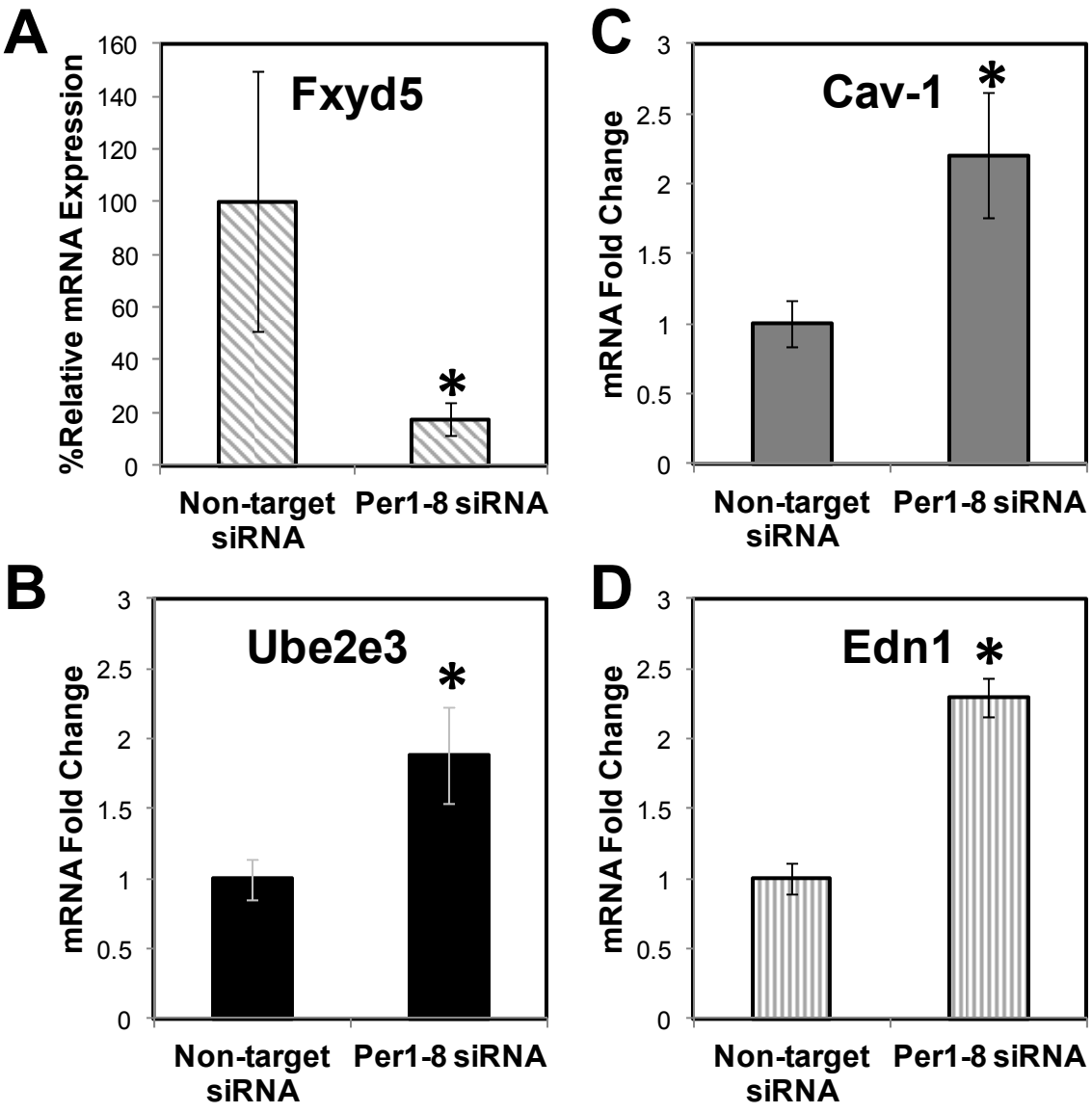

Figure S2. Altered expression of genes involved in sodium transport following Per1 knockdown in mIMCD-3 Cells. mIMCD-3 cells were transfected with a non-target siRNA or Per1 specific siRNA. Under these conditions, Per1 mRNA levels were reduced by about 90%. QPCR was used to measure changes in gene expression following Per1 knockdown for A. Fxyd5, B. Ube2e3, C. Caveolin-1 and D. Edn1.

Supplemental Figure 3

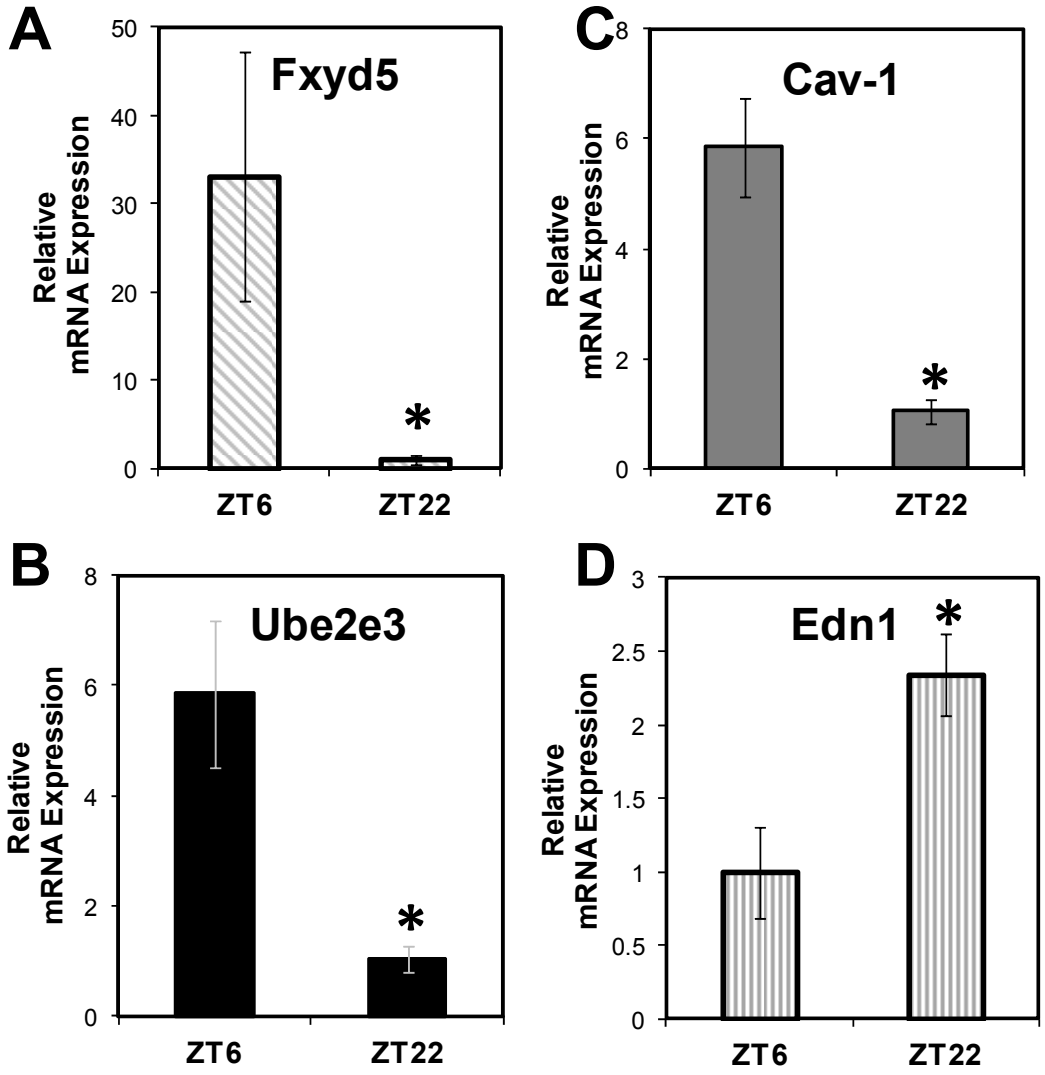

Figure S3. Temporal expression of sodium transport genes in wild type mice. Cortex dissections were made from the kidneys of male wild type (129/sv) mice euthanized at noon (zeitgeiber time (ZT) 6) or 10 pm (ZT22). These samples were previously described[2]. Total RNA was isolated and changes in gene expression were measured using quantitative real time PCR, with values normalized to actin mRNA expression for A. Fxyd5, B. Ube2e3, C. Caveolin-1 and D. Edn1. Fold change values are relative to ZT6. $p<0.05$, n=3-4 animals.

Supplemental Figure 4

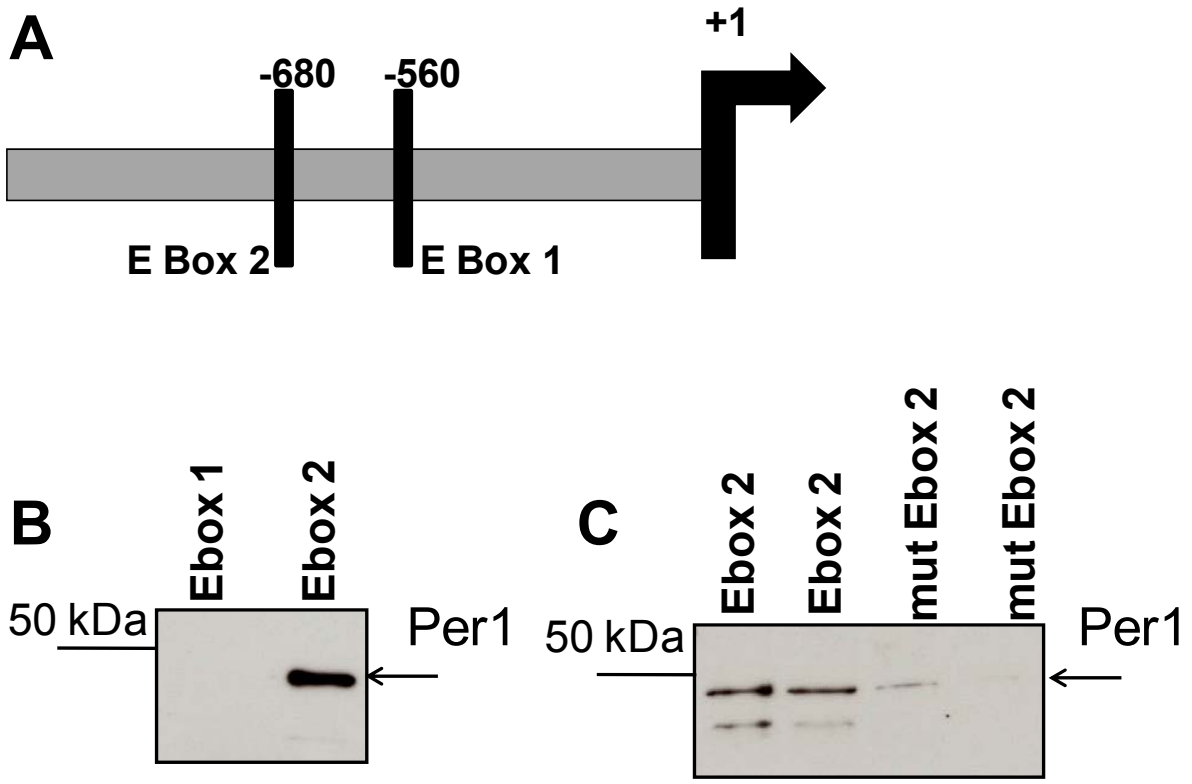

Figure S4. Per1 interacts with an E-box in the *Edn1* Promoter. A. Putative E-box elements in the *Edn1* promoter. TF Search analysis identified two putative E-box response elements in the promoter of the *Edn1* gene. B. Per1 Interacts with E-box 2 as shown by DAPA analysis of putative E-boxes. C. Mutated DAPA with E-box 2 from *Edn1* promoter. The sequence of E-box 2 (agacttggtggaaggggtggtggtggaaaagt) was mutated (agacttggtggaagctctctctgtggaaaagt).

Supplemental Figure 5

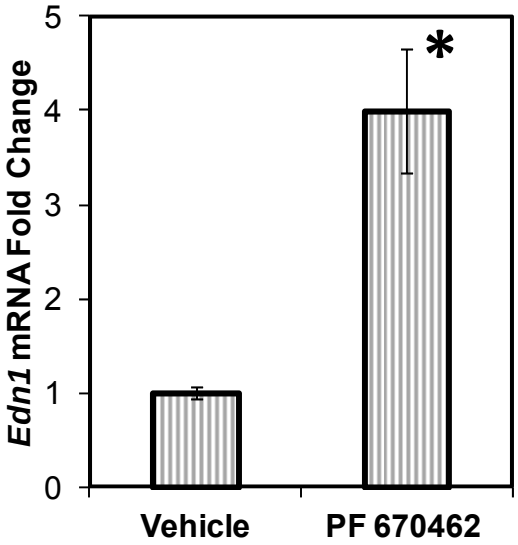

Figure S5. *Edn1* mRNA expression is increased in mpkCCD$_{c14}$ cells treated with Casein Kinase δ/ε inhibitor. Cells were treated with PF670462 (Santa Cruz) or vehicle (DMSO) for 72 h. Gene expression was measured using QPCR, with values normalized to actin mRNA expression. Fold change values are relative to vehicle. *p<0.05, n=6.

Supplemental Figure 6

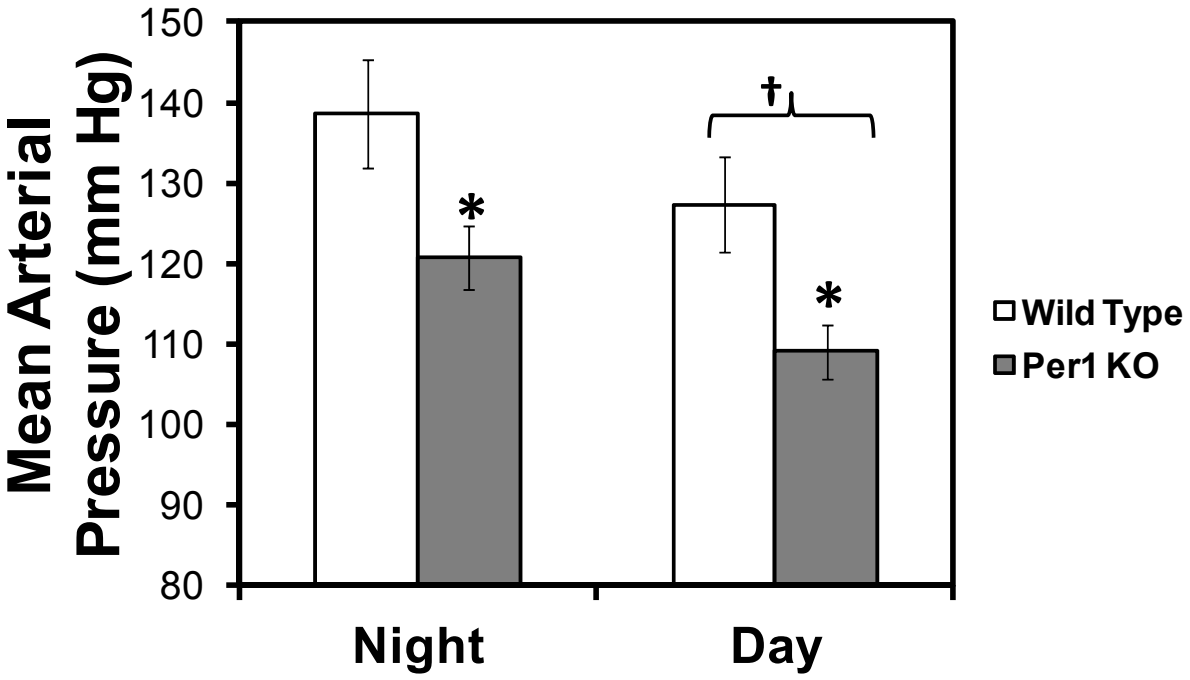

Figure S6. Per1 KO mice exhibit significantly lower BP in the night and day. Data were collected and analyzed as described in the main text, Figure 4. 2-way ANOVA analysis demonstrated statistically significant reductions in mean arterial pressure (MAP) between genotypes (*P<0.05). There was a significant difference due to time in both Per1 KO and WT mice (†P<0.05). There was not a significant interaction between genotype and time.

Supplemental Figure 7

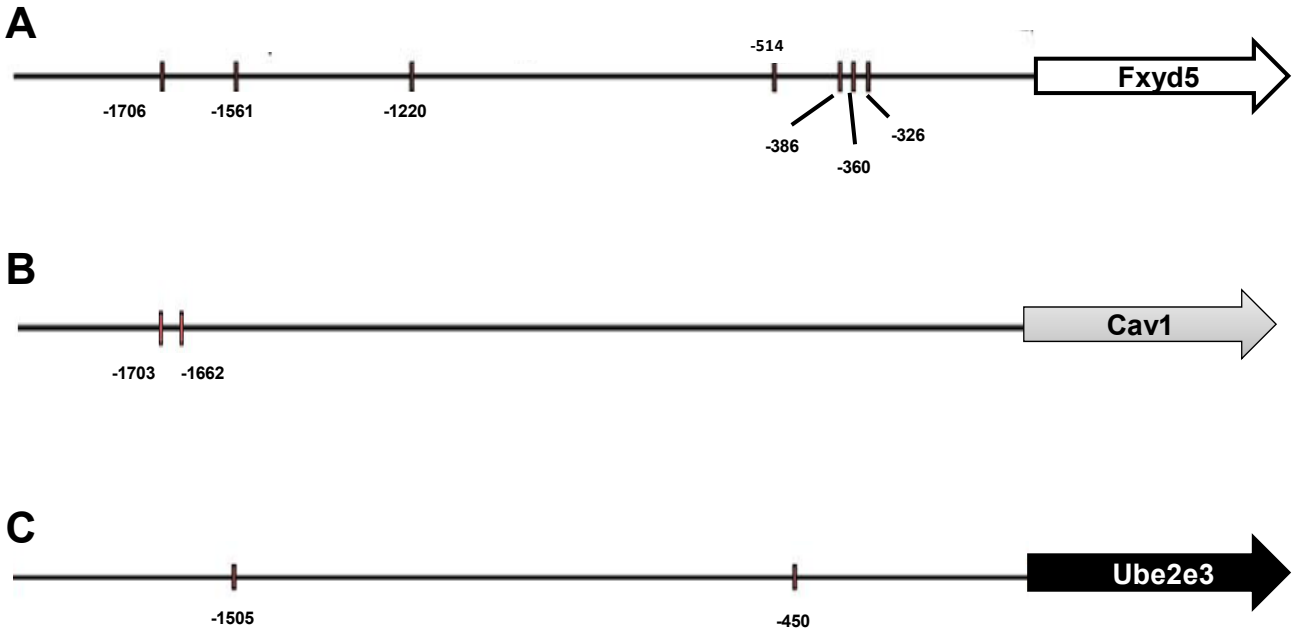

Figure S7. Putative E-boxes in the promoters of Per1 target genes. The promoters for the Per1 target genes *Fxyd5* (Panel A), *Cav1* (Panel B) and *Ube2e3* (Panel C) were evaluated for predicted E-box response elements using PROMO[6, 7] with the threshold set at 85%, 75% and 85%, respectively.