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

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Abstract

The circadian clock protein Period 1 (Per1) contributes to the regulation of expression of the α subunit of the renal epithelial sodium channel (αENaC) at the basal level and in response to the mineralocorticoid hormone aldosterone. The goals of the present study were to define the role of Per1 in the regulation of additional renal sodium handling genes in cortical collecting duct cells and to evaluate BP in mice lacking functional Per1. To determine if Per1 regulates additional genes important in renal sodium handling, a candidate gene approach was employed. Immortalized collecting duct cells were transfected with a non-target siRNA or a Per1 specific siRNA. Expression of the genes for αENaC and Fxyd5, a positive regulator of Na, K-ATPase activity, decreased in response to Per1 knockdown. Conversely, mRNA expression of caveolin-1, Ube2e3 and ET-1, all negative effectors of ENaC, was induced following Per1 knockdown. These results led us to evaluate BP in *Per1* KO mice. Mice lacking Per1 exhibit significantly reduced BP and elevated renal ET-1 levels compared to wild type animals. Given the established role of renal ET-1 in ENaC inhibition and blood pressure control, elevated renal ET-1 is one possible explanation for the lower blood pressure observed in Per1 KO mice. These data support a role for the circadian clock protein Per1 in the coordinate regulation of genes involved in renal sodium reabsorption. Importantly, the lower BP observed in *Per1* KO mice compared to wild type suggests a role for Per1 in BP control as well.

Keywords

kidney; circadian rhythm; clock; collecting duct; gene regulation

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Disclosures. None

Introduction

Approximately one-third of Americans are afflicted with hypertension, the leading risk factor for cardiovascular disease. The majority of these patients suffer from essential hypertension, for which there is no established etiology. Increasing evidence suggests a role for the circadian clock in the control of blood pressure (BP). A subset of hypertensive individuals do not experience the normal nighttime decrease in BP and are at greater risk for cardiovascular complications[1]. These so-called "non-dippers" are known to suffer from increased left ventricular hypertrophy, atherosclerosis, microalbuminuria, congestive heart failure, stroke and myocardial infarction. In addition to this non-dipping phenotype, BP abnormalities and cardiovascular disease are well known in night-shift workers[2,3]. Although these clinical correlations have been established, the underlying molecular mechanisms are poorly understood.

The core circadian clock consists of a positive and negative transcriptional feedback loop. In the positive loop, Bmal1 and Clock drive transcription of the Per (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2) genes. In the negative feedback loop, Per and Cry action inhibit the action of Bmal1 and Clock, thereby decreasing their own transcription[4]. Circadian clock proteins interact with E-box response elements in target gene promoters to affect transcriptional regulation. Although Per1 has been characterized as a transcriptional repressor, increasing evidence suggests that it may participate in transcriptional activation, perhaps in a gene or tissue specific manner[5,6,7].

The circadian clock gene *Per1* is an aldosterone target in renal collecting duct (CD) cells[8]. Per1 contributes to the basal and aldosterone-dependent transcription of the *Scnn1a* gene that encodes the α subunit of the epithelial sodium channel (αENaC)[6]. *Scnn1a* expression was reduced in the renal medulla of *Per1* knockout (KO) mice. Further investigation into the regulation of αENaC by Per1 revealed that cortical αENaC mRNA was reduced in *Per1* KO mice and Per1 knockdown resulted in reduced αENaC protein levels in immortalized murine renal cortical CD (CCD) mpkCCD$_{c14}$ cells[7]. Given the critical role of ENaC in sodium transport and BP control, the results suggest that the clock contributes to circadian fluctuations in sodium excretion and BP.

Expression profiling experiments in different tissues have shown that 6-8% of the genes were subject to circadian control (reviewed in[9]). Temporal analysis of gene expression in the distal convoluted tubule and CCD showed that hundreds of transcripts were expressed in a circadian manner[10]. Given the known circadian oscillations in gene expression in these cell types, we used a model of the CCD to identify novel Per1 targets. The results suggest that Per1 coordinately regulates several genes encoding products that function in renal sodium reabsorption. Finally, we show for the first time that *Per1* KO mice exhibited significantly lower BP compared to wild type (WT) mice.

Methods

Animals

Per1 KO mice (129/sv) were provided by Dr. David Weaver (University of Massachusetts[11]) and maintained by Animal Care Services at UF. WT 129/sv control mice were ordered from Charles River. Animals were maintained on a normal 12hr light:dark cycle and fed normal lab chow (Harlan #2018). Experiments were performed with the approval of UF and VA Medical Center IACUCs and in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Data Sciences International telemetry transmitters were surgically implanted through the left carotid artery, extending into the aortic arch

(according to the method of[12]). Mice (18-20 weeks old) were allowed at least seven days to recover before recordings were made.

Cell culture and molecular biology

Detailed methods are available in the supplemental section, please see http://hyper.ahajournals.org.

Statistical Analysis

Statistical analyses were performed using the Student's t-test in Excel. BP data and tissue ET-1 ELISA data were analyzed using two-way ANOVA (SigmaStat) with the Holm-Sidak test. P values less than 0.05 were considered significant.

Results

Per1 coordinately regulates expression of genes involved in sodium transport

Given the demonstrated regulation of αENaC gene expression by Per1[6,7], we investigated the possibility that Per1 regulated additional genes encoding products that participate in sodium transport or the regulation of sodium transport. We used mpkCCD$_{c14}$ cells as a model of the renal CCD because this cell line is well characterized[13] and has been used extensively to study the regulation of ENaC[14-18]. In addition to αENaC, the sodium transport genes, *Fyxd5, Ube2e3, Cav1* and *Edn1* were identified as potential Per1 targets.

Fxyd5 is a positive effector of the Na, K-ATPase that mediates basolateral sodium transport to the blood stream[19]. Greater than 40% reduction in Fxyd5 mRNA was observed following Per1 knockdown in mpkCCD$_{c14}$ cells (Figure 1A). In contrast to αENaC and Fxyd5, whose gene products function in sodium retention, the expression of three inhibitors of sodium reabsorption was induced following Per1 knockdown. *Ube2e3* encodes an E3 ubiquitin ligase linked to ENaC turnover[20], Caveolin-1 (Cav-1) is involved in endocytosis of ENaC[21], and the endothelin-1 peptide (ET-1, encoded by the *Edn1* gene) inhibits ENaC via a decrease in channel open probability[22,23]. Both Ube2e3 and Cav-1 mRNAs increased more than 2.5x in mpkCCD$_{c14}$ cells following Per1 knockdown (Figure 1B, 1C). ET-1 mRNA was induced nearly 4x in the absence of Per1 (Figure 1D). Significant changes were not observed in expression of the Zinc transporter gene *Slc39a14* following Per1 knockdown (Figure S1).

Since antibodies to Cav-1 and αENaC were readily available, we tested whether Per1-mediated regulation of these genes extended to the level of protein. Membrane protein levels of αENaC protein were reduced in Per1-8 siRNA-transfected cells as compared to the non-target siRNA control (Figure 2A, top panel). Cav-1 membrane protein levels were clearly induced following Per1 knockdown (Figure 2A, bottom panel). ET-1 is a secreted peptide hormone of 21 amino acids encoded by the *Edn1* gene. Because the mpkCCD$_{c14}$ cells were grown in transwell inserts, media was collected from the apical (lumen) and basolateral sides of the cultures. Media samples were collected from cells transfected with a control non-target siRNA or Per1-8 siRNA and evaluated for ET-1 peptide levels. ET-1 peptide levels were significantly greater in the media from mpkCCD$_{c14}$ cells transfected with the Per1-8 siRNA compared to control (Figure 2B).

To test whether the regulation of these novel Per1 target genes occurred in a model of the inner medullary CD, mRNA expression levels of Fxyd5, Ube2e3, Cav-1 and ET-1 were evaluated in mIMCD-3 cells. Similar to the effect of Per1 knockdown in mpkCCD$_{c14}$ cells, Fxyd5 mRNA levels were decreased by more than 50% following Per1 knockdown (Figure

S2A). Similarly, Ube2e3, Cav-1 and ET-1 mRNA were significantly induced in the absence of Per1 (Figures S2B-D).

Temporal regulation of sodium transport genes

Regulation of Fxyd5, ET-1, Cav-1 and Ube2e3 by Per1 suggested that expression of these genes may be clock-controlled *in vivo*. Expression of αENaC and Per1 mRNA follows a circadian pattern of expression in the renal cortex with reduced mRNA levels at zeitgeiber time (ZT) 22 (active phase) versus ZT6 (sleep phase)[6]. Therefore, the levels of Fxyd5, ET-1, Cav-1 and Ube2e3 mRNA expression were tested in WT mice by measuring steady state mRNA expression levels during the light and dark cycles (Figure S3). Significant differences in mRNA levels between two time points are suggestive of clock-controlled regulation[10,24]. Like Per1 and αENaC[6], Fxyd5 levels dramatically decreased at ZT22 relative to ZT6 (Figure S3A). Cav-1 and Ube2e3 expression levels were lower at ZT22 relative to ZT6 (Figures S3B, 3C). In contrast, the circadian pattern of ET-1 mRNA expression was inverted with an increase at ZT22 compared to ZT6 (Figure S3D). Given that Per1 mRNA expression is elevated at ZT6 when ET-1 mRNA levels are low and Per1 knockdown resulted in increased ET-1 mRNA, this result may reflect inhibitory action of Per1 on ET-1.

Per1 negatively regulates ET-1 gene expression

Because renal ET-1 plays a critical role in BP control, the regulation of *Edn1* gene expression by Per1 was evaluated in WT and *Per1* KO mice. Consistent with an inhibitory action of Per1 on *Edn1*, levels of ET-1 mRNA were significantly elevated in the renal cortex of *Per1* KO mice compared to WT control mice (Figure 3).

Regulation of ET-1 mRNA by Per1 suggested that Per1 might act directly on the ET-1 gene (*Edn1*). The *Edn1* promoter was evaluated for putative E-box elements using TF Search (http://www.cbrc.jp/research/db/TFSEARCH.html). Of the predicted response elements, we focused on the E-boxes in the *Edn1* promoter shown in Figure S4A, in part because of the proximity of these elements to the aldosterone response elements previously identified in the *Edn1* promoter[19]. DNA affinity purification assays (DAPA) using mpkCCD$_{c14}$ nuclear extracts were performed to investigate Per1 interaction with these putative E-boxes (Figure S4B). Per1 was detected only at E-box 2 located at position −680. Demonstrating the specificity of this interaction, Per1 bound poorly to a mutated E-box 2 probe (Figure S4C). Because Per1 does not contain an inherent DNA-binding domain, its interaction with E-box 2 is likely facilitated through additional clock proteins, as we have shown previously[7].

To further confirm that ET-1 is a target of Per1, mpkCCD$_{c14}$ cells were treated with the casein kinase I (CKI) δ/ε inhibitor PF670462. Phosphorylation by CKI δ/ε is required for Per1 entry into the nucleus[25]. PF670462 thereby blocks Per1 entry into the nucleus, resulting in phase delays in animal models[26,27]. Inhibition of CKI δ/ε in mpkCCD$_{c14}$ cells resulted in a 4x increase in ET-1 mRNA levels (Figure S5).

Per1 KO mice exhibit a BP phenotype

The apparent coordinate regulation of sodium transport genes by Per1 led to the hypothesis that Per1 contributes to the positive regulation of sodium reabsorption and BP. Therefore, BP was evaluated in *Per1* KO and WT 129/sv mice using an arterial radio-telemetry probe. Similar to previous findings[28], this strain of WT mice displayed a mean arterial pressure (MAP) of 133 mm Hg under control conditions. However, *Per1* KO mice exhibited a significantly lower BP compared to WT mice (115 mm Hg, $p<0.05$), and this was associated with decreases in both diastolic and systolic pressures (Table 1). No differences between WT and *Per1* KO mice were observed in heart rate, pulse or activity (Table S1). Both WT

and *Per1* KO underwent a significant daytime reduction in BP (Figure 4), with *Per1* KO MAP significantly lower during day and night.

Per1 KO mice have increased renal ET-1

Because CD-localized ET-1 is known to regulate BP through an ENaC-dependent mechanism[29,30], we examined renal ET-1 levels in *Per1* KO versus WT mice. ET-1 levels were higher in the inner medulla and cortex of *Per1* KO versus WT mice (Figure 5A and B). Interestingly, and consistent with the known role of renal ET-1 in the BP regulation, ET-1 levels were inversely correlated with day versus night BP values in both *Per1* KO and WT mice (Figure S6).

Discussion

The results of the present study demonstrate the novel finding that *Per1* KO mice exhibit lower BP relative to WT mice, with significantly lower systolic and diastolic pressures. We previously reported that loss of Per1 reduced expression of αENaC and resulted in increased urinary sodium[6]. A candidate gene approach was used to identify additional Per1 target genes involved in the regulation of sodium transport. We show that mRNA levels of the Na, K-ATPase effector Fyxd5 were reduced in Per1 knockdown cells. Furthermore, several negative effectors of ENaC activity, Cav-1, Ube2e3 and ET-1, were induced in response to Per1 knockdown. Importantly, we show for the first time that renal ET-1 peptide levels were elevated in *Per1* KO mice. These results are consistent with a role for the circadian clock in regulation of sodium homeostasis and BP.

Per1 KO animals exhibited an 18 mm Hg decrease in 24hr MAP relative to WT mice. These animals are on a 129/sv background, in which WT mice exhibit higher baseline BP than C57/BL6 WT mice[28]. Interestingly, *Per1* KO mice maintained under normal light:dark conditions exhibited circadian variation in BP which suggests that, under these conditions, Per1 may contribute to the basal regulation of BP rather than temporal control. Reports of BP phenotypes in rodents with circadian clock disruption suggest that the clock is critical for cardiovascular function (reviewed in[31]). Whereas *Clock* KO mice maintained a normal 24hr rhythm of BP, the average MAP and mean systolic BP were significantly lower in *Clock* KO mice compared to WT[10]. Likewise, *Bmal1* KO mice also exhibited lower BP but lacked circadian BP rhythmicity[32]. Elevated aldosterone levels and salt-sensitive hypertension were observed in *Cry1/Cry2* KO mice[33]. Increased activity of Hsd3b6, an enzyme in the aldosterone synthesis pathway, was linked to this phenotype. When maintained on a standard 12hr light:dark cycle, *Per2* mutant mice exhibited decreased 24hr diastolic BP, increased heart rate, and a decreased difference between day and night BP[34]. Under constant darkness, WT mice maintained normal 24hr rhythms in BP, activity and heart rate but *Per2* mutant mice experienced a shortened circadian period. *Per1* KO mice do not display arrhythmic behavior patterns unless they are placed in total darkness[11]. Whether or not total darkness disrupts the circadian pattern of BP in *Per1* KO animals is unknown.

The present finding that *Per1* KO mice display a lower BP than WT mice is consistent with our proposed role for Per1 in the stimulation of sodium reabsorption in the kidney (Figure 6). Moreover, the results demonstrate that Per1 acts as a coordinate regulator of genes encoding products that function in the regulation of sodium reabsorption. We have previously demonstrated that αENaC is positively regulated by Per1[6,7]. The findings that Cav-1, ET-1, Fxyd5 and Ube2e3 appear to be Per1 targets suggest a model in which Per1 and other clock proteins coordinately regulate the expression of sodium transport genes (Figure 6). Importantly, these novel Per1 targets regulate sodium reabsorption at many levels, including ENaC open probability (ET-1), degradation and membrane recycling of ENaC (Ube2e3 and Cav-1), and positive regulation of Na, K ATPase activity (Fxyd5). Like

Edn1, the promoters for *Fxyd5, Ube2e3* and *Cav-1* contain putative E-boxes (Figure S7). A direct link between the circadian clock and ET-1 in the kidney is a particularly intriguing result given that, in contrast to its role as a vasoconstrictor in the vasculature, ET-1 acts as a natriuretic and diuretic hormone in the kidney (reviewed in[35,36]). Kohan and colleagues demonstrated that renal CD-specific ET-1 KO mice exhibit salt-sensitive hypertension[29]. ET-1 represses renal sodium reabsorption, at least in part, via its inhibition of ENaC[22,23] and this effect involves the ET-B receptor[30]. Our observation that inner medullary ET-1 is doubled in Per1 KO mice at midnight is especially intriguing given that the highlest levels of ET-1 in the body are found in the inner medulla[35]. Increased renal ET-1 in *Per1* KO mice is one possible explanation for the lower BP observed in these animals.

Although the Per1 signaling mechanism identified here using a CCD model is consistent with the significantly lower BP observed in *Per1* KO mice, extra-renal effects of Per1 cannot be ruled out because of global Per1 deletion in these animals. For example, the circadian clock regulates vascular function as well. Rudic and colleagues have demonstrated that *Bmal1* KO mice and *Clock* mutant mice display endothelial dysfunction and vascular injury[37]. It is not yet clear if the aberrant vascular function observed in these mice contributes to the lower BP observed in both *Bmal1*[32] and *Clock* KO[10] mice. Tissue-specific deletion of these clock genes may be needed to gain a more complete understanding of the mechanism through which the circadian clock contributes to regulation of cardiovascular function.

Perspectives

The present study demonstrates that *Per1* KO mice display a significantly lower BP than WT mice and that Per1 represses the expression of ET-1, a known inhibitor of renal sodium reabsorption. The coordinate regulation of several key sodium transport genes by Per1 provides further support for a central role of the circadian clock in the regulation of renal function. That Per1 KO mice exhibit significantly lower BP suggests that loss of Per1 may be protective against hypertension. Future studies aimed at identifying how Per1 and the circadian clock regulate BP should shed significant light on the BP disorders so often observed in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

1. What is New?
   - Per1 contributes to the regulation of several genes encoding proteins that regulate renal sodium retention.
   - Mice lacking functional Per1 have a significantly lower BP compared to control mice.
2. What is Relevant?
   - Because sodium handling by the kidney is an important determinant of BP, our observation that Per1 regulates genes involved in sodium transport demonstrates that Per1 may be an important player in the control of BP.
   - Mice lacking Per1 have much lower BP than control mice, indicating that loss of Per1 may be protective against hypertension.
3. Summary: Per1 is an important regulator of gene expression in the kidney and likely plays a critical role in the regulation of BP.

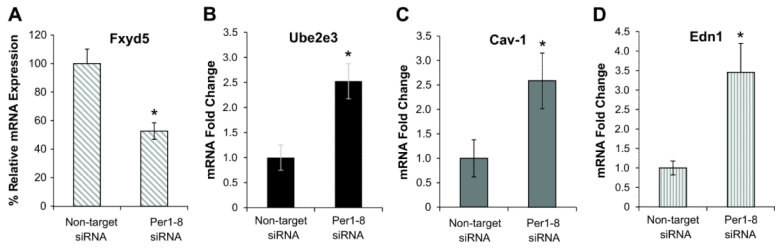

Figure 1. Altered expression of genes involved in sodium transport following Per1 knockdown in mpkCCD$_{c14}$ Cells

mpkCCD$_{c14}$ 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.

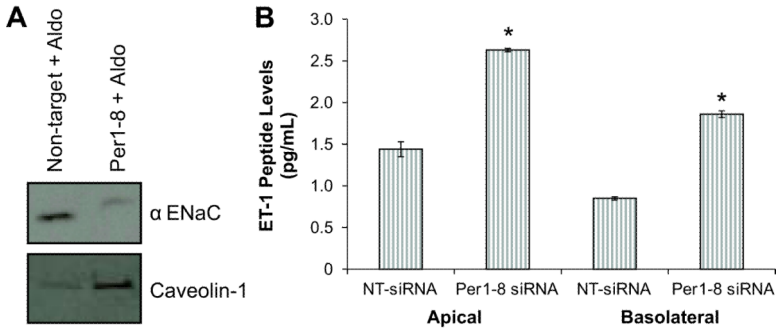

Figure 2. Protein expression levels are affected by Per1 knockdown in mpkCCD$_{c14}$ cells

mpkCCD$_{c14}$ cells were transfected with a non-target siRNA or Per1 specific siRNA. Under these conditions, Per1 mRNA levels are reduced by 80%. **A.** Western blot analysis of membrane protein from aldosterone (Aldo)-treated, siRNA-transfected mpkCCD$_{c14}$ cells was performed using an anti-αENaC antibody or an anti-Caveolin-1 antibody. **B.** ET-1 peptide levels were measured in media samples from cells grown in transwell dishes; top media corresponds to apical and bottom media to basolateral. *$p<0.05$, $n=6$. Data are representative of at least two independent experiments.

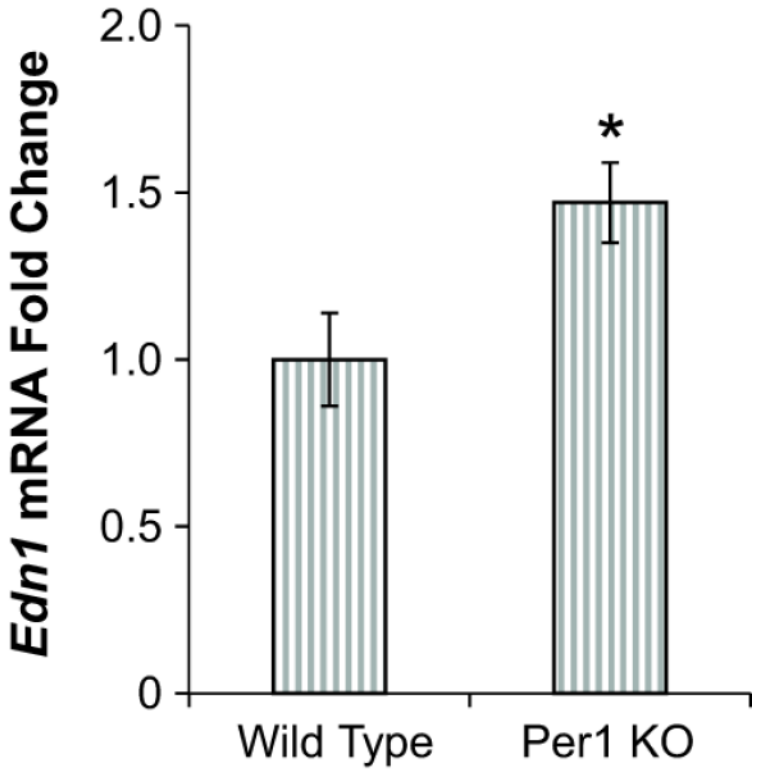

Figure 3. Per1 negatively regulates the expression of ET-1

Edn1 mRNA levels are higher in the renal cortex of Per1 KO mice compared to WT mice. Cortex dissections were made from the kidneys of age-matched, male WT (129/sv) or Per1 KO mice. Gene expression was measured using QPCR, with values normalized to actin mRNA expression. Fold change values are relative to WT. *$p<0.05$, n=6 animals of each genotype.

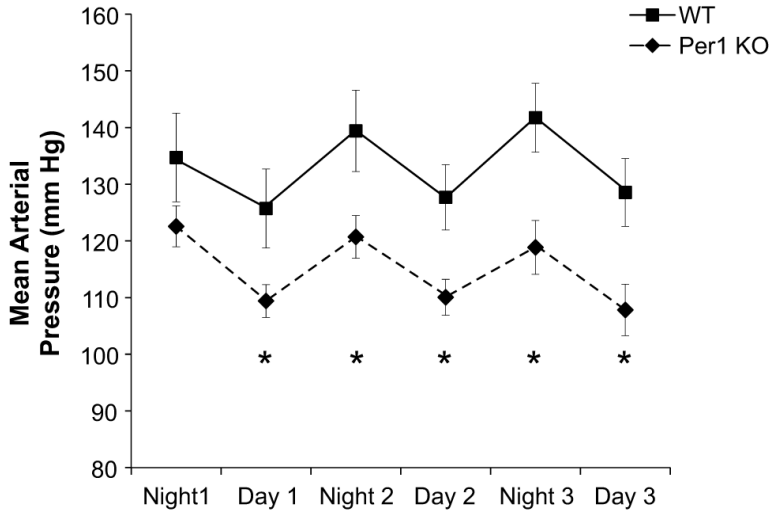

Figure 4. Loss of the circadian clock protein Per1 results in decreased BP

Radio-telemetry recordings were made in Per1 KO and WT mice. MAPs were averaged over each day or night period. *$p<0.05$ for Per1 KO compared to WT at each time point, n=6. Data were also analyzed by 2-way ANOVA (SigmaPlot). Genotype and time effects were significant but there was not a statistically significant interaction between genotype and time.

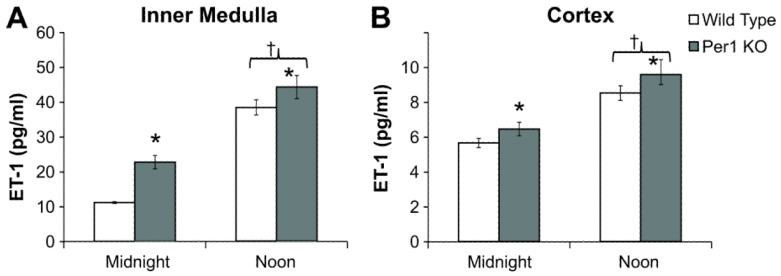

Figure 5. Renal ET-1 peptide levels are elevated in *Per1* KO mice

ELISA was used to measure ET-1 peptide levels in the inner medulla (Panel A, n=4) and cortex (Panel B, n=6) of Per1 KO and WT mice at midnight or noon. Data were analyzed using 2-way ANOVA (SigmaPlot), with significant genotype (*P<0.05) and time (†P<0.05) effects. There was not a statistically significant interaction between genotype and time.

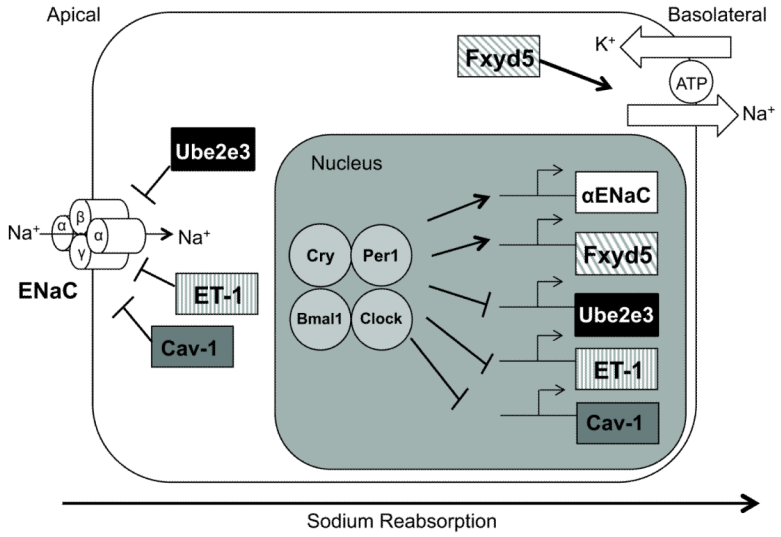

Figure 6. A model for Per1 action on genes involved in sodium transport

Gene expression studies in immortalized murine renal CD cells following Per1 knockdown indicate that Per1 positively regulates αENaC and Fxyd5 mRNA expression while it negatively regulates Ube2e3, ET-1 and Cav-1 mRNA expression. This coordinate regulation of sodium transport genes by Per1 suggests a role for Per1 in transepithelial sodium reabsorption.