The Circadian Protein Period 1 Contributes to Blood Pressure Control and Coordinately Regulates Renal Sodium Transport Genes

Lisa R. Stow, Jacob Richards, Kit-Yan Cheng, I. Jeanette Lynch, Lauren A. Jeffers, Megan M. Greenlee, Brian D. Cain, Charles S. Wingo, Michelle L. Gumz

Abstract—The circadian clock protein period 1 (Per1) contributes to the regulation of expression of the α subunit of the renal epithelial sodium channel 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 blood pressure (BP) in mice lacking functional Per1. To determine whether Per1 regulates additional genes important in renal sodium handling, a candidate gene approach was used. Immortalized collecting duct cells were transfected with a nontarget small interfering RNA or a Per1-specific small interfering RNA. Expression of the genes for α-epithelial sodium channel 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 epithelial sodium channel, was increased after 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 with wild-type animals. Given the established role of renal ET-1 in epithelial sodium channel inhibition and BP control, elevated renal ET-1 is one possible explanation for the lower BP 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 with wild-type mice suggests a role for Per1 in BP control as well. (Hypertension. 2012;59:00-00.) • Online Data Supplement

Key Words: kidney □ circadian rhythm □ clock □ collecting duct □ gene regulation

Approximately one third of Americans are afflicted with hypertension, the leading risk factor for cardiovascular disease. The majority of these patients experience 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 nondippers are known to participate in transcriptional activation, perhaps in a gene- or tissue-specific manner.

The core circadian clock consists of a positive and negative transcriptional 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.

The circadian clock gene Per1 is an aldosterone target in renal collecting duct (CD) cells.5 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

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knockdown resulted in reduced αENaC protein levels in immortalized murine renal cortical CD (CCD) mpkCCDc14 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% to 8% of the genes were subject to circadian control (reviewed in Reference 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 with wild-type (WT) mice.

Methods

Animals

Per1 KO mice (129/sv) were provided by Dr David Weaver (University of Massachusetts11) and maintained by Animal Care Services at University of Florida. WT 129/sv control mice were ordered from Charles River. Animals were maintained on a normal 12 hours light:dark cycle and fed normal laboratory chow (Harlan). Experiments were performed with the approval of University of Florida and Veterans Affairs Medical Center institutional animal care and use committees and in accordance with the National Institutes of Health 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 Reference 12). Mice (18–20 weeks old) were allowed ≥7 days to recover before recordings were made.

Cell Culture and Molecular Biology

Detailed methods are available in the online-only Data Supplement.

Statistical Analysis

Statistical analyses were performed using the Student t test in Excel. BP data and tissue endothelin (ET) 1 ELISA data were analyzed using 2-way ANOVA (SigmaStat) with the Holm-Sidak test. P values <0.05 were considered significant.

Results

Per1 Coordinates 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 mpkCCDc14 cells as a model of the renal CCD because this cell line is well characterized13 and has been used extensively to study the regulation of ENaC.14–18 In addition to αENaC, the sodium transport genes Fxyd5, 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 bloodstream.19 More than 40% reduction in Fxyd5 mRNA was observed after Per1 knockdown in mpkCCDc14 cells (Figure 1A). In contrast with αENaC and Fxyd5, for which gene products function in sodium retention, the expression of 3 inhibitors of sodium reabsorption was induced after 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 ET-1 peptide (encoded by the Edn1 gene) inhibits ENaC via a decrease in channel open probability.22,23 Both Ube2e3 and Cav-1 mRNAs increased >2.5 times in mpkCCDc14 cells after Per1 knockdown (Figure 1B and 1C). ET-1 mRNA was induced nearly 4 times in the absence of Per1 (Figure 1D). Significant changes were not observed in expression of the Zinc transporter gene Slc39a14 after Per1 knockdown (Figure S1, available in the online-only Data Supplement).

Because 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 small interfering RNA (siRNA)-transfected cells as compared with the nontarget siRNA control (Figure 2A, top). Cav-1 membrane protein levels were clearly induced after Per1 knockdown (Figure 2A, bottom). ET-1 is a secreted peptide hormone of 21 amino acids encoded by the Edn1 gene. Because the mpkCCDc14 cells were grown in transwell...
Figure 2. Protein expression levels are affected by period (Per) 1 knockdown in mpk cortical collecting duct (CCD)\(_{c14}\) cells. mpkCCD\(_{c14}\) cells were transfected with a nontarget small interfering RNA (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-epithelial sodium channel α (ENaC) antibody or an anti-caveolin 1 antibody. B, Endothelin (ET) 1 peptide levels were measured in media samples from cells grown in transwell dishes; top medium corresponds with apical and bottom medium with basolateral. *P<0.05, n=6. Data are representative of ≥2 independent experiments.

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 with 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 identified previously in the Edn1 promoter.\(^{19}\) DNA affinity purification assays 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-δ/ε inhibitor
PF670462. Phosphorylation by casein kinase I-δε 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 casein kinase I-δε in mpkCCD14 cells resulted in a 4-times 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 radiotelemetry probe. Similar to previous findings,28 this strain of WT mice displayed a mean arterial pressure of 133 mm Hg under control conditions. However, Per1 KO mice exhibited a significantly lower BP compared with WT mice (115 mm Hg; P<0.05), and this was associated with decreases in both diastolic and systolic pressures (Table). No differences between WT and Per1 KO mice were observed in heart rate, pulse, or activity (Table S1). Both WT and Per1 KO mice underwent a significant daytime reduction in BP (Figure 4), with Per1 KO mean arterial pressure 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 5B). 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).

**Table. Summary of Telemetry Data**

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<th>Per1 KO</th>
<th>P Value</th>
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<td>116.879*</td>
<td>0.009</td>
</tr>
<tr>
<td>Diastolic</td>
<td>126.593</td>
<td>108.096*</td>
<td>0.023</td>
</tr>
<tr>
<td>24 h MAP</td>
<td>132.978</td>
<td>114.902*</td>
<td>0.026</td>
</tr>
</tbody>
</table>

WT indicates wild type; KO, knockout; MAP, mean arterial pressure. *P<0.05 vs WT.

**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 reported previously that loss of Per1 reduced expression of αENaC and resulted in increased urinary sodium.3 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 Reference 31). Whereas Clock KO mice maintained a normal 24-hour rhythm of BP, the average mean arterial pressure and mean systolic BP were significantly lower in Clock KO mice compared with WT mice.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 12-hour light:dark cycle, Per2 mutant mice exhibited decreased 24-hour diastolic BP, increased heart rate, and a decreased difference between day and night BP.34 Under constant darkness, WT mice maintained normal 24-hour rhythms in BP, activity, and heart rate, but Per2 mutant mice experienced a shortened circadian period. Per1 KO mice do not display arrhythmia behavior patterns unless they are placed in total darkness.11

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 demonstrated previously that αENaC is positively regulated by Per1.5,7 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 References 35, 36). Ahn et al29 demonstrated that renal CD-specific ET-1 KO mice exhibit salt-sensitive hypertension. 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 highest 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, extrarenal 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. Anea et al37 have demonstrated that Bmal1 KO mice and Clock mutant mice display endothelial dysfunction and vascular injury. It is not yet clear whether the aberrant vascular function observed in these mice contributes to the lower BP observed in both Bmal1 and Clock KO 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 transport.
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 aim at identifying how Per1 and the circadian clock regulate BP should shed significant light on the BP disorders so often observed in humans.

Sources of Funding
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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

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Supplemental Material

Methods

**Cell culture.** Cells were maintained in DMEM/F12 plus 10% FBS (Invitrogen) and 50 μg/ml gentamicin. The mpkCCD<sub>c14</sub> cells were a kind gift of Dr. Alain Vandewalle † and mIMCD-3 cells were purchased from American Type Culture Collection.

**RNA Silencing.** RNA silencing experiments were performed as described<sup>2</sup>. Cells used for siRNA experiments were seeded at a density of 75,000 cells per cm<sup>2</sup> 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<sup>2</sup>. 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<sup>™</sup> III reverse transcriptase (Invitrogen). Resulting cDNAs (20 ng) were used as templates in duplicate QPCR reactions (Applied Biosystems). Cycle threshold (C<sub>T</sub>) values were normalized against β-actin (actb) and relative quantification was performed using the ΔΔC<sub>T</sub> method<sup>3</sup>. All QPCR experiments were performed with TaqMan® primer/probe sets that have guaranteed 100% PCR efficiency over six logarithms of template<sup>4</sup>.

**Western blot.** Western blot real time was performed as described<sup>5</sup> 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<sub>c14</sub> 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<sub>c14</sub> cells using the NE-PER kit (Pierce) according to the manufacturer’s instruction and subjected to DNA-affinity purification analysis (DAPA) as described<sup>5</sup>. Double stranded DNA probes were biotinylated on each 5’ end (Sigma Genosys) and were homologous to E-box 2: 5’-AGACTTGGTGGGAAGGGGTGGTGGTGGAAAAGT or E-box 1: 5’-GGATGTACCTGACAAAACCACATTGTTGTTTATC in the Edn1 promoter (see Figure 5B). E-box 2 mutant sequence was 5’ AGACTTGGTGGAGCTCTCTCTCTGTGGGAAAAAGT. 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.

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WT: wild type, KO: knockout
Supplemental Results

Supplemental Figure 1.

**Figure S1. Effect of Per1 knockdown on expression of the Zinc transporter Slc39a14.** mpkCCD<sub>c14</sub> 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<sub>c14</sub> cells or B. mIMCD-3 cells.
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.
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 (zeitgeber time (ZT) 6) or 10 pm (ZT22). These samples were previously described. 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.
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 (agacttggtggaagctctctctctctctctctctctgtaaaagt).
Figure S5. Edn1 mRNA expression is increased in mpkCCD<sub>c14</sub> 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.
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.
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.