CLOCK phosphorylation by AKT regulates its nuclear accumulation and circadian gene expression in peripheral tissues

Amelia K. Luciano1, Wenping Zhou2, Jeans M. Santana1, Cleo Kyriakides1, Heino Velazquez3, and William C. Sessa1

Department of Pharmacology1, Cell Biology2 and Vascular Biology and Therapeutics Program (VBT), Yale University School of Medicine, New Haven, CT 06520, USA
Department of Internal Medicine3, VA Connecticut Healthcare System, West Haven, Connecticut

Running Title: Akt phosphorylation of CLOCK

*To whom correspondence should be addressed: William C. Sessa, Ph.D., Vascular Biology & Therapeutics Program, Department of Pharmacology, Yale University School of Medicine, Amistad Research Building, 10 Amistad St, New Haven, CT 06520, USA, Tel: (203)737-2291; Fax: (203)737-2290; E-mail: william.sessa@yale.edu

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Abstract

Circadian locomotor output cycles kaput (CLOCK) is a transcription factor which activates transcription of clock-controlled genes (CCG) by heterodimerizing with BMAL1 and binding to E-box elements on DNA. While several phosphorylation sites on CLOCK have already been identified, this study characterizes a novel phosphorylation site at Serine 845 (S836 in humans). Here we show that CLOCK is a novel AKT substrate in vitro and in cells, and this phosphorylation site is a negative regulator of CLOCK nuclear localization by acting as a binding site for 14-3-3 proteins. To examine the role of CLOCK phosphorylation in vivo, ClockS845A knock-in mice were generated using CRISPR/Cas9 technology. ClockS845A mice are essentially normal with normal central circadian rhythms and hemodynamics. However, examination of core circadian gene expression from peripheral tissues demonstrated that
Clock\textsuperscript{S845A} mice have diminished expression of Per2, Reverba, Dbp and Npas2 in skeletal muscle and Per2, Reverba, Dbp, Per1, Rora and Npas2 in the liver during the circadian cycle. The reduction in Dbp levels is associated with reduced H3K9ac at E-boxes where CLOCK binds despite no change in total CLOCK levels. Thus, CLOCK phosphorylation by AKT on S845 regulates its nuclear translocation and the expression levels of certain core circadian genes in insulin sensitive tissues.

Introduction

The core positive activators of the circadian transcriptional feedback loop are BMAL1, CLOCK, and NPAS2. To activate circadian transcription, BMAL1 heterodimerizes with either CLOCK or NPAS2 through their bHLH-PAS domains (1). Together these transcription factors bind to E-box DNA response elements where they affect transcription of a number of clock-controlled genes (CCG). In fact, 5-10\% of transcripts in any given tissue are estimated to undergo circadian rhythms (2,3). Hence, the activity of these transcription factors must be intricately controlled to maintain the circadian rhythmicity of transcripts.

Circadian rhythms are regulated stringently by post-translational modifications which often follow circadian patterns. Multiple kinases have been shown to phosphorylate circadian transcription factors. For example, GSK3\(\beta\) phosphorylates both positive regulators CLOCK and BMAL1 (4) which reduces their stability and increases transcriptional activity. Additionally, AKT2 phosphorylates BMAL1 at S42 in hepatocytes (5) promoting its cytoplasmic localization. The model states that phospho-BMAL1 either binds the adaptor protein 14-3-3 in the nucleus which exports BMAL1 to the cytosol, and/or this interaction delays BMAL1 re-entry to the nucleus by masking its nuclear localization sequence (5).

CLOCK was the first circadian protein discovered in mammals as an effector of circadian locomotor behavior (6). Neither Clock transcripts nor CLOCK protein levels cycle in all tissues, unlike the other circadian feedback loop regulators (7,8), suggesting that CLOCK activity is more likely regulated post-translationally. Three independent phospho-proteomics screens examining substrates specific to AKT isoform deletion (9) or insulin signaling (10,11) found CLOCK phosphorylation on S845. The role of CLOCK S845 has not been confirmed or tested in vitro or in vivo.

Here we show that CLOCK S845 is phosphorylated in vitro and in cells in an AKT-dependent manner. AKT phosphorylation of CLOCK does not influence CLOCK turnover but generates a 14-3-3 binding site that influences CLOCK nuclear:cytoplasmic shuttling. In order to study the role of CLOCK S845 phosphorylation in vivo, a CRISPR/Cas9 knock-in mouse where the phospho-site serine is mutated to an alanine (CLOCK\textsuperscript{S845A}) was generated. CLOCK\textsuperscript{S845A} mice exhibit decreased hepatic and skeletal muscle circadian gene transcription mid-way through the light period which correlates to the time CLOCK:BMAL1 activation occurs without differences in feeding or activity consistent with data in Clock mutant and null mice (12-15). Therefore, CLOCK S845 phosphorylation site (S845) impacts CLOCK activity and may link nutrient sensing to circadian transcription.
RESULTS

AKT phosphorylates CLOCK S845. A phospho-proteomics screen examining AKT specific substrates in mouse endothelial cells determined that endogenous CLOCK was phosphorylated at S845 (9). This site of CLOCK phosphorylation was also identified by mass spectrometry in two additional phospho-screens investigating insulin responsive phosphorylation sites (10) and PI3K-Akt sensitive sites (11) in NIH3T3-L1 adipocytes. Upon further examination, this site has an AKT substrate motif, RxRxxpS, where x represents any amino acid and pS is a phospho-serine acceptor site. This motif in CLOCK is conserved amongst mammals (Figure 1A), although it is not conserved in lower organisms such as flies implying that this phosphorylation site evolved in complex organisms to regulate intricate aspects of circadian rhythms.

To experimentally validate CLOCK S845 phosphorylation by AKT, several independent approaches were used. Initially, in vitro kinase assays were performed using immuno-isolated, activated Flag-AKT, a 16-amino acid CLOCK peptide surrounding the phospho-site or immunoprecipitated wild type (WT) CLOCK or CLOCK S845A proteins as substrates. Flag-AKT phosphorylated the CLOCK peptide and phosphorylation was eliminated in the peptide containing the S845A mutation (Figure 1B). Using either a pCLOCK S845 antibody (validated in Figure 1C) or 33P ATP incorporation into immuno-isolated CLOCK (Figure 1E, lane 3), WT CLOCK was phosphorylated by AKT, and phosphorylation was reduced in the CLOCK S845A mutant. The faint recognition of S845A CLOCK with the pCLOCK S845 antibody but the elimination of 33P ATP incorporation implies that this is due to contamination of the antibody with total anti-CLOCK antibody during the negative selection process. Next, HEK293T cells were transfected with Myc-CLOCK, treated with an allosteric inhibitor of AKT, MK2206, and CLOCK S845 phosphorylation of immunoprecipitated CLOCK examined. Inhibition of AKT reduced CLOCK phosphorylation on S845 as assessed using a p-AKT substrate motif antibody (Figure 1D, left) or the pCLOCK S845 antibody (Figure 1D, middle and quantified in right panel). Finally, stimulation of the PI3K-Akt signaling axis with platelet derived growth factor (PDGF) in NIH3T3 cells induced time-dependent CLOCK phosphorylation after immunoprecipitation of CLOCK, as detected with the pCLOCK S845 antibody (Figure 1F). pCLOCK S845 levels were not detectable in whole cell lysates implying that the level of CLOCK phosphorylation is low or that the antibody has a low titer and/or affinity for this site. Collectively, these data demonstrate that CLOCK S845 is a novel AKT substrate in vitro and in cells.

CLOCK S845 phosphorylation does not influence its stability but regulates nuclear translocation via binding to 14-3-3. To investigate the role for CLOCK S845 phosphorylation in cells, several experiments were conducted. Since phosphorylation can regulate protein stability and turnover, CLOCK stability (Myc-tagged WT and S845A CLOCK) after transfection into HEK293T cells was examined after blocking protein synthesis with cycloheximide (CHX) or proteasomal degradation with MG132. Neither CHX nor MG132 treatment had a differential effect on WT or S845A CLOCK levels after transfection (Figure 2A and B). Since AKT phosphorylation of transcription factors such as FOXO1 regulates its subcellular distribution, we tested whether phosphorylation of CLOCK impacted its nuclear localization. Expression of CLOCK in HEK293T cells, followed by quantitative imaging demonstrated that approximately 25% of WT CLOCK is localized to the
nucleus as compared to 50% of CLOCK S845A (Figure 3A and quantified in B; p<0.001). Inhibition of AKT with MK2206 increased nuclear localization of WT CLOCK to similar levels as CLOCK S845A, but did not impact the levels of nuclear CLOCK S845A (p<0.001). These data imply that phosphorylation of S845 is important for the cytoplasmic:nuclear shuttling of CLOCK.

Many AKT substrates, especially transcription factors such as those of the FOXO family (16) and BMAL1 (5), bind to 14-3-3 proteins, thereby preventing their nuclear localization, altering their conformation, or increasing their ability to bind with other proteins. The 14-3-3 family preferentially binds to one of two canonical phosphorylated motifs or a less defined non-canonical motif. The region surrounding CLOCK S845 (RHRTDSLTDTP) is predicted to be a canonical Arg-containing phospho-motif for 14-3-3 binding by protein prediction programs such as the Eukaryotic Linear Motif (ELM) resource. To test this possibility, HEK293T cells were transfected with Myc-tagged WT or S845A CLOCK and Myc tagged proteins immunoprecipitated then blotted for associated 14-3-3. Immunoprecipitation of Myc-tagged WT CLOCK resulted in greater co-precipitation of 14-3-3 than did Myc-tagged CLOCK S845A (Figure 3C for blots and quantification below). Residual binding of 14-3-3 to CLOCK S845A may be due to 14-3-3 binding to one of the other five predicted 14-3-3 binding motifs or through BMAL1 which also binds 14-3-3 proteins after AKT phosphorylation (5).

CLOCK S845A mice are viable and healthy. To study the importance of CLOCK S845 in vivo, CRISPR/Cas9 technology was employed to generate CLOCK S845A knock-in mice. Mutant mice were generated with a single allele where the codon for serine 845 (AGC) was mutated to alanine (GCC) (Figure 4A). Additionally, to prevent the guide RNA recognizing the donor template, a single base pair synonymous substitution (cytosine to thymine) was engineered into the corresponding PAM sequence, resulting in an unchanged leucine residue just after the mutation site. The top three most likely off-target mutations were sequenced from genomic DNA template, and only those mice without off-target mutations were used for breeding. To generate the mice used for experiments, the ClockS845A heterozygous mice were backcrossed 1-2 generations to wild-type C57Bl/6J mice. After backcrossing, ClockS845A heterozygotes (Hets) were crossed to produce Hets for breeding, and F2 WT and ClockS845A homozygote (CLOCK S845A) mice used for experiments. Upon genotyping greater than 100 pups, the observed frequencies of each genotype were as expected (Figure 4B). Thus, this mutation does not affect development or viability.

Over the course of a year, both male and female (Figure 4C) mice were identical in body weight. In addition, the following parameters were not different in littermate WT mice versus CLOCK S845A mice: basal body temperature (Figure 4D), body composition (Figure 4E), food consumption (Figure 4F), and activity (Figure 4G). There was no change in VCO2 (Figure 4H) or VO2 (Figure 4I); however, there was lower respiratory exchange ratio (RER) (Figure 4J, p<0.001) in CLOCK S845A mice from approximately ZT5 to ZT12 (1PM to 7PM) suggesting a metabolic preference for fatty acid utilization over carbohydrates. Cardiovascular hemodynamics were also examined, and no differences were found in mean arterial pressure, systolic blood pressure, diastolic blood pressure, pulse pressure, heart rate, and activity when the data were analyzed as 3-hour averages throughout the day (Figure 5A-F, respectively).
CLOCK S845A mice do not have central circadian rhythm locomotor or behavioral defects, but have altered circadian gene expression in peripheral tissues. There is conflicting evidence as to whether CLOCK plays a role in regulation of central circadian rhythms as measured by locomotor activity throughout the day. Clock homozygous mutant mice with a deletion of exon 19 are arrhythmic and do not maintain behavioral circadian rhythms (6,17) but Clock KO mice do not have a locomotor rhythm defect (18). Thus, to examine the impact of S845 CLOCK phosphorylation on locomotor rhythms, central circadian rhythms were examined. A representative actogram from a WT and a S845A mouse is shown in Figure 6A. Overall there were no differences in the body weight (Figure 6B), food intake (Figure 6C), tau (period) (Figure 6D), activity onset (Figure 6E) length of active phase (alpha) (Figure 6F), or activity levels/hour (Figure 6G) of S845A mice compared to WT littermate mice. Thus, CLOCK S845A mice are similar to CLOCK KO mice and do not show a locomotor phenotype (18).

Although there was no defect in central locomotor rhythms, CLOCK activity was assessed in peripheral tissues that undergo rhythmic circadian gene expression (13). Thus, transcriptional rhythms of core circadian genes were investigated in the heart, skeletal muscle, and liver. WT and CLOCK S845A mice were fed ad libitum and sacrificed at four time points throughout the day: ZT1 (8AM), ZT5 (12PM), ZT13 (8PM), and ZT18 (1AM). The transcription of E-Box controlled circadian genes such as Per2, Reverba, Dbp and REV and ROR controlled genes Bmal1 and Npas2 were not different in heart extracts from the two strains, however Clock expression was lower in CLOCK S845A mice (Figure 7A). In skeletal muscle, the expression of Reverba and Dbp (at ZT 6) and Npas2 (at ZT1) were reduced (Figure 7B). Interestingly, the most well-characterized organ influenced by peripheral circadian rhythms, the liver (19,20), demonstrated a marked reduction in E-box (CLOCK) regulated genes Per2, Reverba, and Dbp in CLOCK S845A mice (Figure 8A). Circadian genes controlled mainly by REV and ROR responsive elements (RREs), such as Npas2, Bmal1, and Clock did not show a trend in expression differences between the two genotypes and only Npas2 was reduced at ZT1 in CLOCK S845A mice.

Since CLOCK S845A mice had reduced levels of Dbp in skeletal muscle and liver, we examined the ability of CLOCK S845 to bind with two regions of the Dbp gene, one promoter region lacking an E-Box and one intronic E-Box element in Dbp regulated by CLOCK/BMAL1 (21,22). Chromatin immunoprecipitation (ChIP) of CLOCK or H3K9ac (a marker of active chromatin) on the Dbp promoter region without E-boxes was not different (Figure 8B) nor was binding to Dbp E-boxes in WT or CLOCK S845A mice (Figure 8C). However, ChIP of H3K9ac on the Dbp E-boxes (where CLOCK binds) showed significantly decreased H3K9ac at both ZT4 and ZT16 (Figure 8C, p<0.05). Therefore, the loss of CLOCK phosphorylation of S845 reduces Dbp expression through decreased acetylation of chromatin but not via differences in CLOCK binding with DNA.

DISCUSSION

The central finding of this study is that phosphorylation of CLOCK at S845 can regulate CLOCK function in vitro and in vivo. These results are concordant with recent studies showing that BMAL1 can be similarly phosphorylated by AKT (5), demonstrating that the AKT signaling pathway has multiple mechanisms to regulate the activity of central components of the circadian clock. The present data
shows that AKT inhibits CLOCK function in cells directly through phosphorylation on S845 which diminishes its nuclear localization. This new mechanism is complementary with AKT phosphorylation of GSK3β indirectly regulating CLOCK function (4,23). In vivo, knock-in mutation of S845 to eliminate phosphorylation at this site does not produce a central circadian rhythm phenotype but influences the expression of circadian controlled genes in peripheral tissues. The lack of a central circadian rhythm phenotype in CLOCK S845A mice is consistent with data in Clock KO mice that also do not have a locomotor rhythm defect (18), presumably due to the presence of NPAS2 as a redundant binding partner for BMAL1.

Previous work using endothelial cells isolated from AKT1 or AKT2 deficient mice, demonstrated that AKT1 to a greater extent than AKT2, can phosphorylate endogenous CLOCK at S845. However, since AKT2 is not as highly expressed or functionally important in endothelial cells compared to AKT1, the phosphorylation by AKT2 was not detectable in this experimental setup. Work by others in adipocytes using targeted proteomics have shown that insulin induces CLOCK S845 phosphorylation (11). This result in conjunction with our data showing that circadian gene expression is altered in insulin sensitive tissues (skeletal muscle and liver) in CLOCK S845A mice implies that regulation of CLOCK phosphorylation of S845 in vivo is most likely via AKT2, the dominant isoform found in insulin sensitive tissues (24). In addition to multiple AKT isoforms, other AGC- family kinases that phosphorylate a similar motif, such as PKA, may phosphorylate CLOCK as well. PKA is activated by forskolin, one of the key stimuli that triggers rhythmic core circadian gene transcription in cell culture (25); thus, it may play a role in CLOCK S845 phosphorylation.

PKA is also activated by glucagon which is secreted during fasting at the beginning part of the light phase, around the time when there is decreased transcription of CLOCK controlled genes in the liver. Clearly, additional experiments are needed to dissect the relationship between these kinases, CLOCK S845 phosphorylation, and circadian gene expression.

In order to validate CLOCK as an AKT substrate, many of the above experiments were completed in HEK293T cells and NIH3T3 cells; however, the interactions described are not cell autonomous. Mechanistically, when AKT phosphorylates CLOCK at S845, approximately half of the total CLOCK shifts its localization from the nucleus to the cytoplasm, similar to that seen when BMAL1 and FOXO transcription factors are phosphorylated by AKT. pCLOCK binds to 14-3-3 proteins and is prevented from dephosphorylation and re-entering the nucleus. Thus, by altering subcellular localization, the phosphorylation of CLOCK at S845 can regulate the expression of clock-controlled genes as demonstrated in CLOCK 845A mutant mice.

Paradoxically, S845A mice have reduced transcription of multiple E-box regulated genes during the day in livers and skeletal muscle although phosphorylation of AKT substrates is likely not maximal at these time points. This may be due to its phosphorylation in vivo by other kinases, such as PKA, as mentioned above. In attempts to monitor S845 phosphorylation in vivo using multiple approaches, we were unable to detect the peptide containing CLOCK S845 by mass spectrometry due to technical issues, and therefore, do not have a clear time course of pCLOCK S845 in tissue throughout a circadian cycle. However, this phospho-peptide has been detected in previous studies in vivo (26,27) lending
One question that remains is why CLOCK S845A causes reduced histone acetylation of Dhp at the intronic E-box element. There is evidence that CLOCK itself has intrinsic histone acetyltransferase (HAT) activity (28); however, we were unable to detect HAT activity in recombinant CLOCK to measure whether its enzymatic activity is altered in the CLOCK S845A mutant protein. Also, it is possible that phosphorylation of CLOCK S845 alters the recruitment or activity of canonical HATs or deacetylases to chromatin. Perhaps the decreased histone acetylation precedes and causes reduced gene expression. If this was the case, there would be lower levels of nuclear CLOCK over the dark cycle.

In summary, we have characterized CLOCK as a novel AKT substrate that can regulate CLOCK localization in vitro and core circadian gene expression in vivo. Additional work examining metabolic control of this pathway in disease models influenced by circadian rhythms will further enable our understanding of the importance of CLOCK S845 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**- CLOCK (5157), MYC (2276), AKT (pan) (2920), pAKT T308 (9275), pAKT S473 (9271), 14-3-3 (8312), pAkt substrate motif (9614), and GAPDH (5174) antibodies were purchased from Cell Signaling Technologies. The pCLOCK S845 antibody was made for our lab by Cell Signaling Technologies’ product development group. Briefly, rabbits were injected with a KLH conjugated phosphopeptide (PRHRTDSLTDPSKV, mouse sequence with underlined S delineating phospho-site) and antibody purified on columns with an immobilized immunogen and a non-phosphopeptide of the same motif. The HSP90 (sc-69703) antibodies was from Santa Cruz (Dallas, TX, USA). FLAG-M2 antibody (F1804) was from Sigma (St. Louis, MO, USA). β-Actin (ab8226), CLOCK (ChIP grade, ab3517) and H3K9ac (ChIP grade, ab3441) antibodies were purchased from Abcam. Secondary antibodies were fluorescent conjugated donkey anti-mouse, rabbit, or goat from Rockland Immunochemicals (Pottstown, PA USA) (800nm) or Life Technologies (Carlsbad, CA, USA) (680nm).

**Cell Culture**- All HEK293T and NIH3T3 cells were cultured in 10% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin in DMEM (Gibco). All cells were grown in cell culture dishes at 37°C and 5% CO2. The cells were then genotyped, and WT and CLOCK S845A cells passage 4-20 were used in experiments. MK2206 (Selleck Chem) was used at a 5μM.

**Plasmids**- The Myc-CLOCK constructs were gifts from Dr. John Hogenesch. The phospho-site mutation in Myc-CLOCK was made using the TagMaster Mutagenesis system (GM Bioscience, Fredrick, MD, USA). Flag-Akt1 was created in a pFLAG-CMV2 backbone.

**Western Blotting**- Western Blots were done following a standard protocol and imaged on the Odyssey imaging system by LI-COR (Lincoln, NE, USA). Due to low level immunoreactivity of the pCLOCK antibody, blots were first probed with the pCLOCK antibody and then re-probed afterwards with the total CLOCK antibody. Westerns were quantified using the Odyssey imaging software to measure band intensity of protein of interest over total protein or loading control protein (GAPDH or β-actin).

**Immunoprecipitation (IP)**- Cell lysates with incubated with antibodies overnight at 4°C in IP buffer (20mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton-X 100, 10mM β-
mercaptoethanol, 150mM NaCl, 1mM EDTA, 1 mM EGTA, and 1% Na-deoxycholate) with protease and phosphatase inhibitors. The next day, the appropriate Protein A or G Sepharose beads (Millipore, Billerica, MA, USA) were rotated in each IP for 2 hours at 4˚C; then the beads were washed at least 3 times in IP buffer and boiled in 2 x SDS loading buffer to dissociate the protein from the beads.

**Immunofluorescence**- HEK293T cells were transfected with Myc-CLOCK WT or Myc-CLOCK S845A. Cells were blocked with 5% BSA and stained with CLOCK antibody (1:500) overnight. AlexaFluor goat anti-rabbit 568nm at a 1:2000 dilution was used for the secondary antibody (Thermo Fisher, Waltham, MA, USA). Nuclei were stained with ToPro3 (Thermo Fisher, Waltham, MA, USA). Images were taken on the Leica SP5 confocal microscope (Wetzlar, Germany) and analyzed using ImageJ. Nuclei were outlined and measured in ImageJ by hand tracing. CLOCK staining in the nuclei was traced and measured by ImageJ. Then CLOCK staining in the whole cell was traced and measured. The percentage in the nucleus was calculated as the CLOCK staining area in the nucleus divided by total CLOCK staining area multiplied by 100.

**In Vitro Kinase Assays**- Immuno-isolated Flag-AKT1 was added at 5 nM to reactions containing 5-20 μM of peptide (United Peptide, Herndon, VA, USA) or protein immunoprecipitated from in vitro translation reactions. The reaction contained 0.3 μCi/μl of ATP (γ-33P) and 100 μM cold ATP. Reactions were carried out up to 30 min at 30˚C. To quench the peptide reaction, peptides were put on P81 filter paper and dropped into phosphoric acid solution. Protein substrate reactions were put in 2x SDS loading buffer and were boiled. Proteins were then separated by SDS-PAGE. Radioactivity was visualized with Phosphor Screen and Phosphor Imager. Western blot was used in non-radioactive samples to visualize CLOCK and pCLOCK S845.

**Animals**- Clock S845A knock-in mice were made on a C57Bl/6 background using CRISPR/Cas9 technology with the help of the Yale Animal Genomics Facility. The guide RNA had the sequence: 5’TGTAATACGACTCATATAGGGAGCGTTCAGGTTCGGCACAGGACTGACGCCACAGTAGCAGACACACTCTCTCTGACATGCAAAGGAG-3’. The DNA template strand had the sequence: 5’-TCGCACCACCCAGCAACACAGACACACTCTCTCTGACATGCAAAGGAG-3’. Mouse genomic DNA was checked for the top 3 most probable off-target mutations, and only those mice without detected off-target mutations were used for breeding. Mice were genotyped using 2-step amplification qPCR and either the WT forward primer: 5’-GGCACAGGACTGACAG-3’ or the S845A forward primer: 5’-GCACAGGACTGACGC-3’, and the same reverse primer: 5’-CTCATCAAGGGACTGAAC-3’. The threshold cycle values were at least 6 cycles different. Mice were housed in the Yale Animal Facility, fed normal chow, and were kept at 25˚C on a 12 hr LD cycle. All mice used in experiments were at least the F2 generation and were male between 12 and 20 weeks old unless otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee of Yale University.

**qRT-PCR**- RNA was extracted using RNeasy kit (Qiagen). Reverse transcription was completed using the TaqMan Reverse Transcription reagents (Roche). Primers were ordered from the Keck Oligonucleotide Facility (Yale University) from sequences
found on PrimerBank (Harvard University) or published literature as indicated in Table 1. Gapdh was used as the reference gene to normalize RNA quantity.

Telemetry-Telemeter implantation and data collection was completed by Dr. Heino Velazquez as previously described (30). Briefly, heart rate, blood pressure, and activity measurements were collected every minute for at least 5 days. Data was analyzed by first averaging the data into 3-hour bins.

Circadian Wheel Running Assay- Mice 12-16 weeks of age were transferred to individual cages with running wheels attached to monitors (Actimetrics), and data was recorded by ClockLab software (Coulbourn Intruments). After 14 days of acclimation to 12hr light followed by 12hr dark (LD), the mice were kept in complete darkness (DD) for 14 days. Revolutions per minute data was collected, and all data was analyzed using the ClockLab software.

Body temperature measurements- Mouse body temperature was measured by anal probe until the electronic thermometer read an equilibrated temperature. The mice were restrained by scruffing during this process. Temperature readings taken during the dark phase of the cycle were done under red light.

Metabolic measurements-12-16 week old male mice were housed individually in metabolic cages for three days during which VO₂, VCO₂, activity, energy expenditure, respiratory exchange ratio, food intake, and water intake were measured. Their weight was taken and body composition (%fat/%muscle) was measured using a TD-NMR minispec (Bruker).

Chromatin Immunoprecipitation (ChIP)- Mice were sacrificed at ZT4 and ZT16; livers were snap frozen and stored at -80°C. ChIP was carried out as previously described (31). Chromatin was incubated overnight, rotating at 4°C with the following ChIP grade antibodies: CLOCK, H3K9ac, or IgG control antibody (Abcam). The IP was completed and washed with 5 buffers of varying salt concentrations to enhance specificity. Finally, DNA/protein complexes were eluted, samples were reverse crosslinked, and DNA was purified. qPCR was carried out on different areas of the circadian gene Dbp using the primers in Table 1. Data was expressed as “% Input” by comparing the maximum amount of input DNA to the levels in the IP samples.

Data analysis- All experiments were performed at least 3 times; numbers of mice used in each experiment are described in the figure legends. Immunofluorescence was analyzed in ImageJ. mRNA cycling data was analyzed using JTK_Cycle (32). Wheel running data was quantified and analyzed using ClockLab. All other data was analyzed using GraphPad Prism (La Jolla, CA, USA). Graphs comparing only two columns of data were compared using an unpaired Student’s t-test. Graphs with more than two columns were compared using a two-way ANOVA. Data are presented as mean ± s.e.m.
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Author Contributions: AKL designed and conducted most of the experiments and wrote the manuscript. WZ designed and conducted in vitro experiments and JMS aided in completing the experiments. CK helped with the RNA isolations, qPCR, and genotyping. WCS supervised the overall project and wrote manuscript.

Conflict of Interest: The authors declare no conflicts of interest.

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Figure legends:

**Figure 1:** AKT phosphorylates CLOCK in vitro. (A) The C-terminus of CLOCK has a conserved RxRxxS phosphorylation motif in mammals. (B) A 16-amino acid peptide starting with the -5 arginine (R840 in mouse) is phosphorylated by AKT in vitro. (C) Myc-CLOCK WT or Myc-CLOCK S845A was immunoprecipitated from transfected HEK293T cells. Immunoblotting with the pCLOCK S845 antibody demonstrates specificity of the antibody. There was minor binding with CLOCK S845A, but the antibody binds more strongly with phosphorylated CLOCK S845. (D) Myc-CLOCK WT was transfected into HEK293T cells and either treated with vehicle (DMSO) or Akt inhibitor MK2206 (5 μM, 2 hours). The pAkt substrate motif antibody and the pClock S845 antibody bind strongly with the untreated Myc-CLOCK as quantified to the right (mean ± s.e.m. n = 3. *p<0.05. (E) In vitro kinase assay with immuno-isolated CLOCK and AKT shown by Western Blot (top panel) and autoradiography with gamma 33-P-ATP (bottom panel). (F) Stimulation of NIH3T3 fibroblasts with PDGF-BB (50 ng/mL) induces phosphorylation of CLOCK S845, shown using a phospho-site specific antibody. This experiment is representative of at least 3 independent experiments and is quantified (pClock/total Clock) to the right. In panels D and F, immunoprecipitated CLOCK was first blotted for p-CLOCK, then re-probed for total CLOCK levels. Unless noted, data are presented as mean ± s.e.m. n= 3. *p<0.05

**Figure 2:** CLOCK stability and degradation are not affected by phosphorylation at S845. (A) HEK293T cells were transfected with Myc-CLOCK and Myc-CLOCK S845A and treated with cycloheximide (CHX). Quantification of 4 experiments, where total CLOCK was normalized to GAPDH loading control, is shown on the right. Graph is presented as mean ± s.e.m, n= 4 experiments. (B) HEK293T cells were transfected with Myc-CLOCK WT and Myc-CLOCK S845A and treated with MG132. Graph is presented as mean ± s.e.m, n= 3 experiments.

**Figure 3:** Phosphorylation on S845 regulates nuclear CLOCK localization and its interaction with 14-3-3. (A) Immunofluorescence of Myc-CLOCK WT or Myc-CLOCK S845A transfected into HEK293T cells treated with either vehicle or Akt inhibitor MK2206 (5μM). (B) Quantification with ImageJ of nuclear localization calculated by the percentage of CLOCK in the nucleus (co-localization with nuclear stain ToPRO3) compared to the rest of the CLOCK staining within the cell, averaged from greater than 20 cells per experiment, n = 5 individual experiments. (C) Co-immunoprecipitation of either Myc-CLOCK WT or S845A with endogenous 14-3-3 proteins (pan 14-3-3 antibody) from lysates of HEK293T cells. n = 3. Data are presented at mean ± s.e.m. *** p< 0.001.

**Figure 4:** CLOCK S845A knock-in mice are viable and healthy. (A) Sequencing demonstrating a mouse heterozygous for the CLOCK S845A mutation. The wild-type (WT) sequence has an AGC codon for a serine, while the S845A allele has a GCC encoding an alanine. The highlighted portion of the sequencing data shows two visible peaks for the AG→GC mutation of one allele. Just after this codon, there is silent mutation of a cytosine (bold, blue) to a thymine (red) in the mutant which still encodes for leucine. (B) The percentage of each genotype
from over 100 pups of heterozygous mice mating pairs; the percentages of observed to expected mice were similar. (C) The weight of WT (black) and S845A (red) mice over time. Males are represented by a solid line; females are the dotted line. n = 4-5 mice per timepoint. (D) Body temperature in 12-16 week old male mice throughout the daily cycle, where ZT0 represents lights on. n = 3-4 mice per time point. (E-J) 16 week old S845A male mice and WT littermates were used for metabolic cage experiments. (E) Body weight and body composition of WT and S845A mice. (F) Feeding patterns for WT (black) and S845A (red) over the course of a day. (G) Activity patterns throughout the day. (H) Volume of CO₂ emitted and (I) volume of O₂ consumed. (J) Respiratory exchange ratio (VCO₂/VO₂) throughout the day. Data are represented as mean ± s.e.m. ZT, zeitgeber time, or time after lights on. The bar at the bottom represents the light and dark cycle.

**Figure 5:** CLOCK S845A mice do not have cardiovascular defects. No significant differences were found between WT (black) and S845A (red) mice in: mean arterial pressure (A), systolic blood pressure (B), diastolic blood pressure (C), pulse pressure (D), heart rate (E), and activity (F). n = 3 mice per genotype. Data represented as mean of 3 hours of measurements ± s.e.m.

**Figure 6:** CLOCK S845A mice do not have a circadian locomotor phenotype. (A) Double plotted representative actogram for wheel running activity. Each horizontal line represents 24 hours. The light:dark schedule is shown by 12 hours lights on with yellow background and 12 hours lights off with white background. Where the yellow background ends represents when the mice were subjected to complete darkness (DD). There are no differences in behavior under DD in S845A mice as compared to WT mice. (B) Body weight at the beginning of the study (Day 1) and at the end (Day 28). (C) Total food intake over the course of 28 days of the study. (D) Tau, or period, of activity under complete darkness (DD). (E) Average time of activity onset measured in hours after subjective lights on. (F) Average active period length. (G) Average amount of activity during the active phase. n = 6 WT, 8 S845A. Data is represented as individual points for each mouse with a line at the mean ± s.e.m.

**Figure 7:** Circadian gene expression over a 24-hour period in (A) heart and (B) skeletal muscle (gastrocnemius) from WT and CLOCK S845A mice. The top row of genes in each section (Per2, Reverba, Dhp) are those that are mainly controlled by E-box elements, while the bottom row of each section (Bmal1, Npas2, Clock) are those that are largely controlled by Rev and Ror response elements. Time on the x-axis is given as zeitgeber time (ZT) (time after lights on). The rectangle at the bottom of the figure represents the timing of lights on (white) or lights off (black). n = 3 mice per timepoint. Data are represented as mean + s.e.m. Statistics were calculated using a two-way ANOVA with Bonferroni post-hoc test.

**Figure 8:** Circadian genes controlled by E-boxes have lower expression levels in livers at the middle of the light period (Per2, Dhp, Reverba, Per1). Circadian gene expression in WT and S845A livers is shown in each bar graph for the genes indicated on the y-axis of each graph. The black and white bar at the bottom represents the light and dark schedule over the course of a day. Data is represented as mean ± s.e.m. * p<0.05. n = 3 mice per timepoint. (B and C) Chromatin immunoprecipitation of liver chromatin in mice sacrificed at ZT4 and ZT16 with either Clock or H3K9ac antibody. IgG was used as a background control. (B) ChIP using a primer pair for a Dhp promotor region without an E-box and (C) a primer pair surrounding the
Dbp intronic E-boxes. H3K9ac is decreased at Dbp E-boxes in S845A livers as compared to WT livers. Data is presented as mean ± s.e.m. *p<0.05, n ≥3.
**Fig. 2**

### A

| Myc-CLOCK  | CHX (hrs) | WT | S845A |
|------------|-----------|----|-------|
| Myc        | 0         | 8  | 16    | 24   | 32 |
| GAPDH      | 0         | 8  | 16    | 24   | 32 |

**B**

| Myc-CLOCK  | MG132 (hrs) | WT | S845A |
|------------|-------------|----|-------|
| Myc        | 0           | 3  | 6     |
| β-actin    | 0           | 3  | 6     |
Fig. 3
Fig. 4
Fig. 6
Fig. 7
Fig. 8
CLOCK phosphorylation by AKT regulates its nuclear accumulation and circadian gene expression in peripheral tissues
Amelia K Luciano, Wenping Zhou, Jeans M. Santana, Cleo Kyriakides, Heino Velazquez and William C. Sessa

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