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Phosphorylation of LSD1 by PKCα Is Crucial for Circadian Rhythmicity and Phase Resetting

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SUMMARY

The circadian clock is a self-sustaining oscillator that controls daily rhythms. For the proper circadian gene expression, dynamic changes in chromatin structure are important. Although chromatin modifiers have been shown to play a role in circadian gene expression, the in vivo role of circadian signal-modulated chromatin modifiers at an organism level remains to be elucidated. Here, we provide evidence that the lysine-specific demethylase 1 (LSD1) is phosphorylated by protein kinase Cα (PKCα) in a circadian manner and the phosphorylated LSD1 forms a complex with CLOCK:BMAL1 to facilitate *E-box*-mediated transcriptional activation. Knockin mice bearing phosphorylation-defective *Lsd1*$^{SA/SA}$ alleles exhibited altered circadian rhythms in locomotor behavior with attenuation of rhythmic expression of core clock genes and impaired phase resetting of circadian clock. These data demonstrate that LSD1 is a key component of the molecular circadian oscillator, which plays a pivotal role in rhythmicity and phase resetting of the circadian clock.

INTRODUCTION

The circadian clock regulates daily oscillations and controls many physiological and behavioral systems. In mammals, molecular oscillators located in the suprachiasmatic nucleus (SCN) in the brain, constitute the master clock (Welsh et al., 2010). However, the oscillators are located not only in the SCN, but also in most peripheral tissues for proper regulation of circadian rhythmicity (Mohawk et al., 2012). The core circadian system consists of an interacting transcriptional-translational feedback loop of clock genes. CLOCK and BMAL1 are basic helix-loop-helix (bHLH)-PAS transcription factors and activate the transcription of *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes through binding to *E-box* elements as heterodimers (Gekakis et al., 1998). This CLOCK:BMAL1-mediated transcription is, in turn, inhibited by PER and CRY proteins, which form a negative-feedback loop (Shearman et al., 2000). In addition to this core loop, BMAL1 expression is rhythmically regulated by the orphan nuclear receptors including RORα and REV-ERBα/β (Yang et al., 2006b).

Dynamic changes of chromatin structure are critical events for the proper timing and extent of circadian gene expression. Rhythmic histone modifications have been shown to be associated with the cyclic transcription of several clock-controlled genes, including *Dbp*, *Per1*, and *Per2* (Etchegaray et al., 2003). CLOCK itself has an intrinsic histone acetyltransferase activity, which is required for circadian function (Doi et al., 2006). SIRT1 histone demethylase binds CLOCK and controls the deacetylation of PER2 and BMAL1 as well as histones (Asher et al., 2008). In addition to histone acetylation, histone methylation status has been implicated in circadian clock regulation. MLL1 histone methyltransferase specifically promotes trimethylation of H3K4 for transcriptional activation of circadian target genes (Katada and Sassone-Corsi, 2010). EZH2 histone methyltransferase interacts with CLOCK:BMAL1 complexes and is recruited to the *Per* gene promoters, where it catalyzes the trimethylation of H3K27 for transcriptional repression (Etchegaray et al., 2006). Jarid1a histone demethylase inhibits histone deacetylase 1 (HDAC1) function and enhances CLOCK:BMAL1-mediated

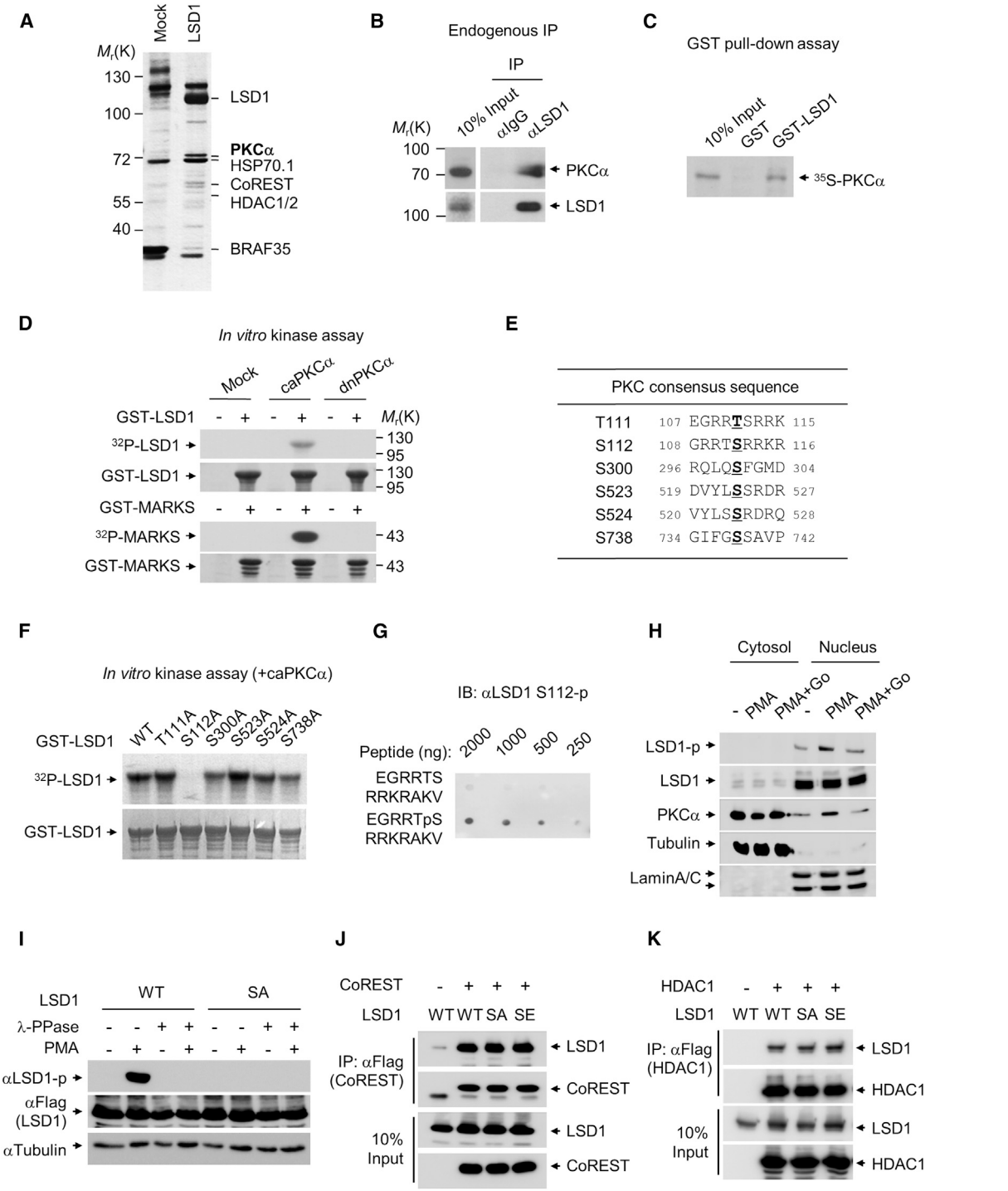

Figure 1. Identification of PKCα-Dependent Phosphorylation of LSD1

(A) LSD1-interacting proteins were purified from HEK293T cells expressing Flag-LSD1 by performing coimmunoprecipitation with Flag M2 agarose. Bound proteins were resolved by SDS-PAGE and prepared for LC-MS/MS analysis.

(B) Coimmunoprecipitation of endogenous LSD1 with PKCα in MEFs.

(C) GST pull-down assay was performed using in-vitro-translated ^{35}S-methionine-labeled PKCα with GST-LSD1.

(D) In vitro kinase assays using a constitutively active form of PKCα (caPKCα) or a kinase-deficient form of PKCα (dnPCKα) immunoprecipitated from cell lysates as the kinase and GST-LSD1 WT or GST-MARKS (as positive control) purified from *E. coli* as substrates were performed. The reaction samples were subjected to 10% SDS-PAGE, and phosphorylated LSD1 was detected by autoradiography.

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transcription in a demethylase-independent manner (DiTacchio et al., 2011).

Growing numbers of reports indicate that posttranslational modification of nonhistone proteins including core clock proteins is important for transcriptional regulation of circadian clock genes as in the case of histone modification (Cardone et al., 2005). Protein kinase Cα (PKCα) has been closely linked to circadian rhythms, because the phenotype of *PKCα*-deficient mice show an impairment of light-mediated clock resetting (Jakubcakova et al., 2007). PKCα is highly expressed in SCN master clock and is responsible for phosphorylation of several core circadian clock proteins including CLOCK and BMAL1 (Robles et al., 2010; Shim et al., 2007; Van der Zee and Bult, 1995). PKCα-mediated CLOCK phosphorylation enhances recruitment of CLOCK to the *Per1* promoter and plays a role in phase resetting (Shim et al., 2007). In addition to phosphorylation, ubiquitination of clock proteins is important. E3 ubiquitin ligase-dependent ubiquitination of CRY has been shown to be important for determination of circadian period of the clock (Yoo et al., 2013).

LSD1 is the first reported lysine-specific, FAD^+-dependent histone demethylase (Shi et al., 2004). LSD1 participates in gene repression process as a part of the REST corepressor (CoREST) complexes mediating the demethylation of H3K4me1/2 (Lee et al., 2005). LSD1 is also involved in gene activation process associated with nuclear receptors through demethylation of H3K9me1/2 (Metzger et al., 2005; Wang et al., 2009). Recent studies reported that LSD1 demethylates nonhistone proteins such as p53, Dnmt1, as well as histones for elaborate regulation (Huang et al., 2007; Wang et al., 2009). Further, LSD1 interacts with SIRT1, a NAD^+-dependent deacetylase, which is known to play important roles in circadian clock (Mulligan et al., 2011).

Although it has been well established that posttranslational modifications of transcription factors and chromatin modifiers can modulate the protein functions by altering the chromatin state or protein-protein interactions, it remains largely unknown how physiological signals regulate the in vivo function of chromatin modifiers at an organism level (Cheung et al., 2000). Here, we identify a critical role of LSD1 at the crossroads between PKC signaling pathways and epigenetic regulation of circadian clock by facilitating CLOCK:BMAL1-mediated transcriptional regulation through PKCα-mediated phosphorylation of LSD1. $Lsd1^{SA/SA}$ knockin mice show a defect in circadian rhythmicity with attenuated rhythmic expression of core clock genes in SCN and impaired phase resetting of circadian clock. These data provide a conceptual and mechanistic link between PKC signaling and epigenetic regulation of circadian rhythmicity and phase resetting.

RESULTS

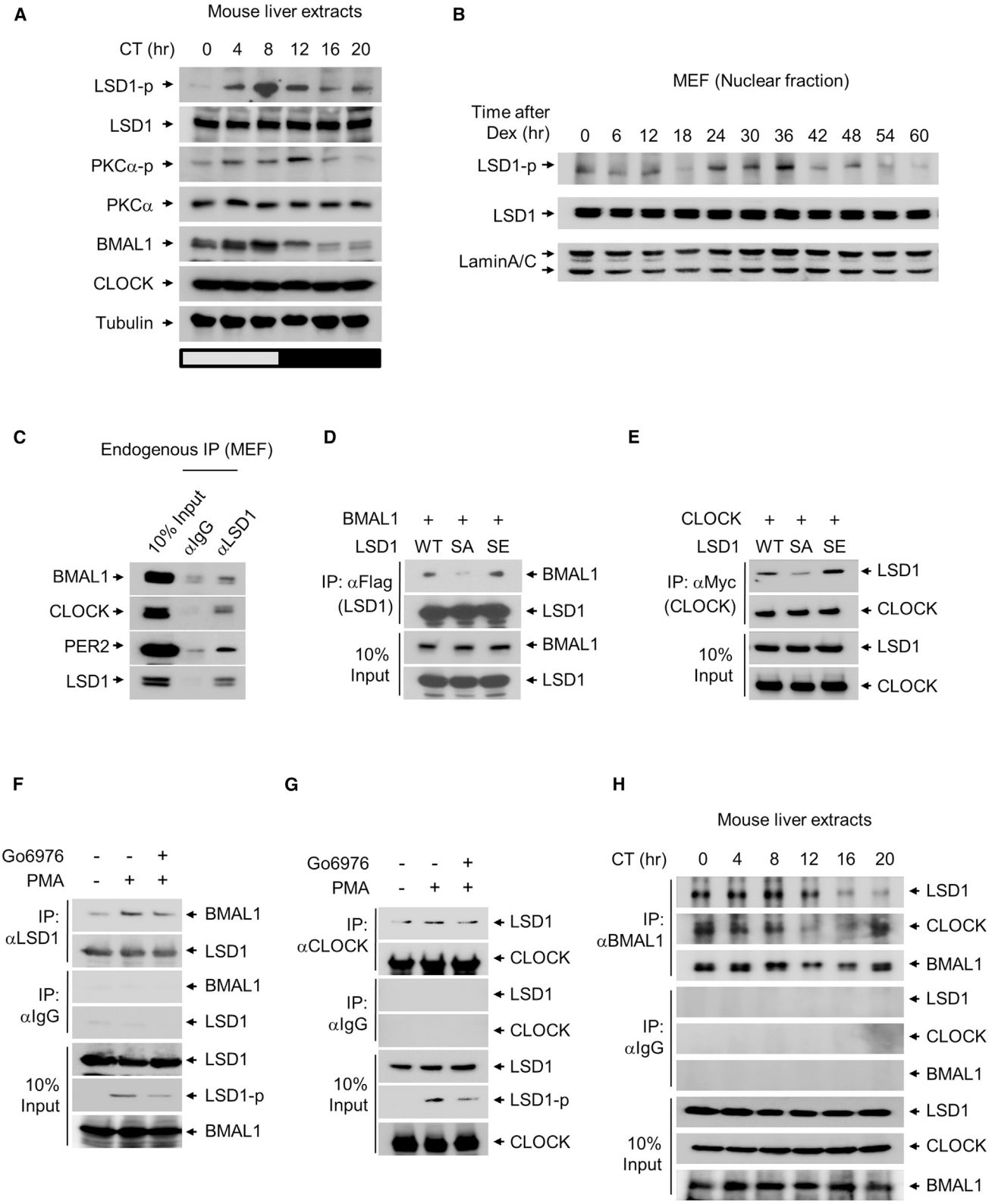
LSD1 Is Phosphorylated by PKCα on Serine 112

Based on the importance of LSD1, which demethylates histone H3 on Lys 4 or Lys 9 for transcriptional repression or activation, respectively (Metzger et al., 2005; Shi et al., 2004), we asked which regulatory signaling factors dynamically modulate LSD1 function and the selection of specific binding partners. We used a FLAG epitope-tag strategy followed by liquid chromatography-tandem mass spectrometry/mass spectrometry (LC-MS/MS) to find LSD1-binding proteins, which are responsible for elaborating LSD1 functions. We detected CoREST, BRCA2-associated factor 35 (BRAF35), Hsp70.1, and HDAC1/2, consistent with previously reported binding partners of LSD1 (Figure 1A; Table S1 available online) (Lee et al., 2005). Unexpectedly, we identified PKCα as an LSD1-interacting protein (Figure 1A; Table S1). The association between LSD1 and PKCα at endogenous expression level was confirmed by coimmunoprecipitation assay (Figure 1B). Glutathione S-transferase (GST) pull-down assay validated direct association of LSD1 with PKCα in vitro (Figure 1C).

The unexpected finding of PKCα as an LSD1-binding protein led us to examine whether PKCα is responsible for direct phosphorylation of LSD1. Therefore, we performed in vitro kinase assay using a constitutively active form of PKCα (caPKCα) or a kinase-defective form of PKCα (dnPKCα) immunoprecipitated from cell lysates. The immunoprecipitated materials from either caPKCα or dnPKCα were incubated with bacterially expressed and purified GST-LSD1 proteins. We found that caPKCα, but not dnPKCα, phosphorylated purified LSD1 proteins (Figure 1D). To determine the potential phosphorylation sites targeted by PKCα in LSD1, the full-length amino acid sequence of mouse LSD1 was entered into the NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK/), and six PKC phosphorylation sites were predicted (Figure 1E). These serine/threonine residues were individually mutated to alanine. In vitro kinase assay revealed that only substitution of serine 112 with alanine (S112A) abrogated PKCα-mediated phosphorylation of LSD1 (Figure 1F).

The specific antibody for phospho-LSD1 S112 was generated using phospho-LSD1 S112 peptide. The antibody recognized the phosphorylated peptide exclusively, as assessed by dot blot analysis (Figure 1G). Using anti-phospho-LSD1 antibody, we examined whether PKC signal activation or inhibition affects LSD1 phosphorylation on S112. Immunoblot analysis by anti-phospho-LSD1 antibody revealed that treatment of phorbol-12-myristate-13 acetate (PMA), a PKC activator, induced nuclear localization of PKCα and increased phosphorylation of endogenous LSD1, whereas treatment of Go6976, a PKC inhibitor, abrogated PMA-induced-LSD1 phosphorylation (Figure 1H).

(E) Identification of six putative PKC phosphorylation sites in LSD1.
(F) In vitro kinase assays were performed as in (D). The six putative phosphorylation sites were individually mutated to alanine, and only S112A mutant abrogated PKCα-mediated phosphorylation in vitro.
(G) The specificity of the antibody raised against phospho-LSD1 S112 peptide was assessed by dot blot analysis.
(H and I) Treatment of Go6976 (H) or λ-phosphatase (I) abolished PMA-dependent phosphorylation of LSD1 on S112 site as assessed by immunoblot analysis with anti-phospho-LSD1 antibody.
(J and K) LSD1 WT, SA mutant, and SE mutant exhibited similar binding affinity to CoREST and HDAC1.
See also Table S1.



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Treatment of λ-phosphatase almost completely eliminated the PMA-induced phosphorylation of LSD1 on S112 as assessed by immunoblot using anti-phospho-LSD1 antibody (Figure 1I). Together, these data demonstrate that LSD1 is directly phosphorylated by PKCα on S112. LSD1 wild-type (WT), SA mutant, and SE mutant exhibited similar binding affinity to CoREST and HDAC1, well-known binding partners of LSD1 (Figures 1J and 1K).

PKCα-Dependent LSD1 Phosphorylation Oscillates in a Circadian Manner

Accumulating reports indicate that PKCα is involved in controlling the circadian timing system. Several PKC isoforms including PKCα exhibit strong expression in the SCN, where the master circadian pacemaker resides (Van der Zee and Bult, 1995). Pharmacological PKC inhibitors and activators evoke phase advances of neuronal rhythms in SCN slice cultures (Schak and Harrington, 1999). It has been shown that genetic abrogation of PKC signaling leads to impaired resetting of the circadian clock (Jakubcakova et al., 2007).

Based on the reports suggesting that PKC signaling forms an integral part of the circadian clock feedback loop (Robles et al., 2010), we examined whether PKCα-mediated phosphorylation of LSD1 plays a role in circadian clockworks. To investigate whether LSD1 phosphorylation is involved in circadian rhythm, we examined the temporal expression of LSD1 phosphorylation in mouse livers. Immunoblot analysis showed that LSD1 phosphorylation was accumulated to the maximal levels reaching at around circadian time (CT) 8–12 in mouse livers (Figure 2A). However, total protein levels of LSD1, measured at 4 hr intervals around the clock, were not changed in mouse livers. Further, we found overlapping phosphorylation patterns of LSD1 and PKCα over a circadian time course (Figure 2A), indicating in vivo correlation of LSD1 phosphorylation with PKCα activation in liver tissues.

We also wanted to establish whether LSD1 phosphorylation exhibits circadian rhythm in mouse embryonic fibroblasts (MEFs) as in the case of mouse liver tissues. Therefore, we treated cells with dexamethasone (Dex), a synthetic glucocorticoid analog commonly used to trigger circadian gene transcription in a variety of cell lines. LSD1 phosphorylation exhibited circadian oscillation in MEFs entrained by a brief application of Dex (Figure 2B). In order to examine whether LSD1 phosphorylation might influence circadian gene expression, we checked the oscillation patterns of *Per2* mRNA level in LSD1 WT- or S112A mutant-reconstituted *Lsd1*$^{-/-}$ MEF after Dex treatment. Intriguingly, the amplitude of *Per2* mRNA oscillation was damped in *Lsd1*$^{-/-}$ MEFs (Figure S1A). LSD1 WT-, but not SA mutant-, reconstituted *Lsd1*$^{-/-}$ MEFs restored oscillation of *Per2* mRNA level in circadian manner after Dex treatment (Figure S1A). These results indicate that PKCα-mediated LSD1 phosphorylation is responsible for the regulation of circadian clock gene expression.

We examined whether LSD1 physically associates with the core clock components at endogenous expression levels in MEFs. Coimmunoprecipitation assays showed that LSD1 bound to canonical clock proteins, such as BMAL1, CLOCK, and PER2 that are known to regulate *E-box*-mediated transcriptions (Figure 2C). To examine whether LSD1 phosphorylation alters binding specificity to core clock proteins, we performed an immunoprecipitation assay with LSD1 WT, SA mutant, or SE mutant to compare binding specificity to BMAL1 and CLOCK proteins. Compared to LSD1 WT, SE mutant exhibited increased binding to BMAL1 and CLOCK, whereas SA mutant showed attenuated binding to BMAL1 and CLOCK (Figures 2D and 2E). Furthermore, PMA treatment increased the interaction between endogenous LSD1 and BMAL1 or CLOCK, whereas Go6976 treatment reduced their interactions (Figures 2F and 2G). These results indicate that LSD1 phosphorylation increases binding affinity of LSD1 toward BMAL1 and CLOCK. We checked the binding profile of LSD1 to CLOCK:BMAL1 over a CT course and found that the binding of LSD1 to CLOCK:BMAL1 occurs during the active phase (Figure 2H), which is overlapping with PKCα phosphorylation patterns over a CT course as shown in Figure 2A.

Lsd1$^{SA/SA}$ Knockin Mice Show a Defect in Circadian Rhythmicity

To examine roles of LSD1 phosphorylation in vivo, we generated *Lsd1*$^{SA/SA}$ knockin mice. To replace serine 112 of LSD1 with alanine in mouse whole genome, we designed a knockin mutant targeting vector, which contained two mutated DNA sequences (AGC → GCC) in the first exon and a loxP-flanked puromycin resistant (Puro^r) cassette in intron. Serine to alanine substitution was introduced by site-directed mutagenesis, which disrupted the NaeI site (Figure 3A). The knockin of *Lsd1*$^{SA/SA}$ was confirmed by both NaeI digestion and sequencing of PCR products using allele-specific primers (Figure 3B; data not shown). No lethality was associated with the targeted *Lsd1*$^{SA/SA}$ alleles, because *Lsd1*$^{SA/SA}$ mice were normal from birth until adulthood and largely indistinguishable from their WT or heterozygote littermates in viability and fertility exhibiting a ratio expected for a

Figure 2. PKCα-Dependent LSD1 Phosphorylation Oscillates in Circadian Manner

(A) Immunoblot analysis was performed in mouse liver extracts with indicated antibodies. Mouse livers were collected for 4 hr intervals in a DD cycle at indicated CT time.

(B) MEFs were synchronized by a dexamethasone shock (1 μM) for 2 hr, and nuclear extracts were collected at 6 hr intervals. Total LSD1 protein and phosphorylated LSD1 protein levels were analyzed by immunoblot. Lamin A/C was used as a loading control for nuclear proteins.

(C) Coimmunoprecipitation assay of endogenous LSD1 with BMAL1, CLOCK, or PER2 in MEFs.

(D and E) Coimmunoprecipitation assays of LSD1 WT, SA mutant, and SE mutant with BMAL1 (D) or CLOCK (E) in HEK293T cells.

(F and G) The interaction between endogenous LSD1 and BMAL1 or CLOCK was increased after PMA treatment but Go6976 treatment attenuated PMA-induced increased bindings in HEK293T cells.

(H) The binding profile of LSD1 to BMAL1/CLOCK in mouse livers over a circadian time course. Mouse livers were collected for 4 hr intervals in a DD cycle at indicated CT time.

See also Figure S1.

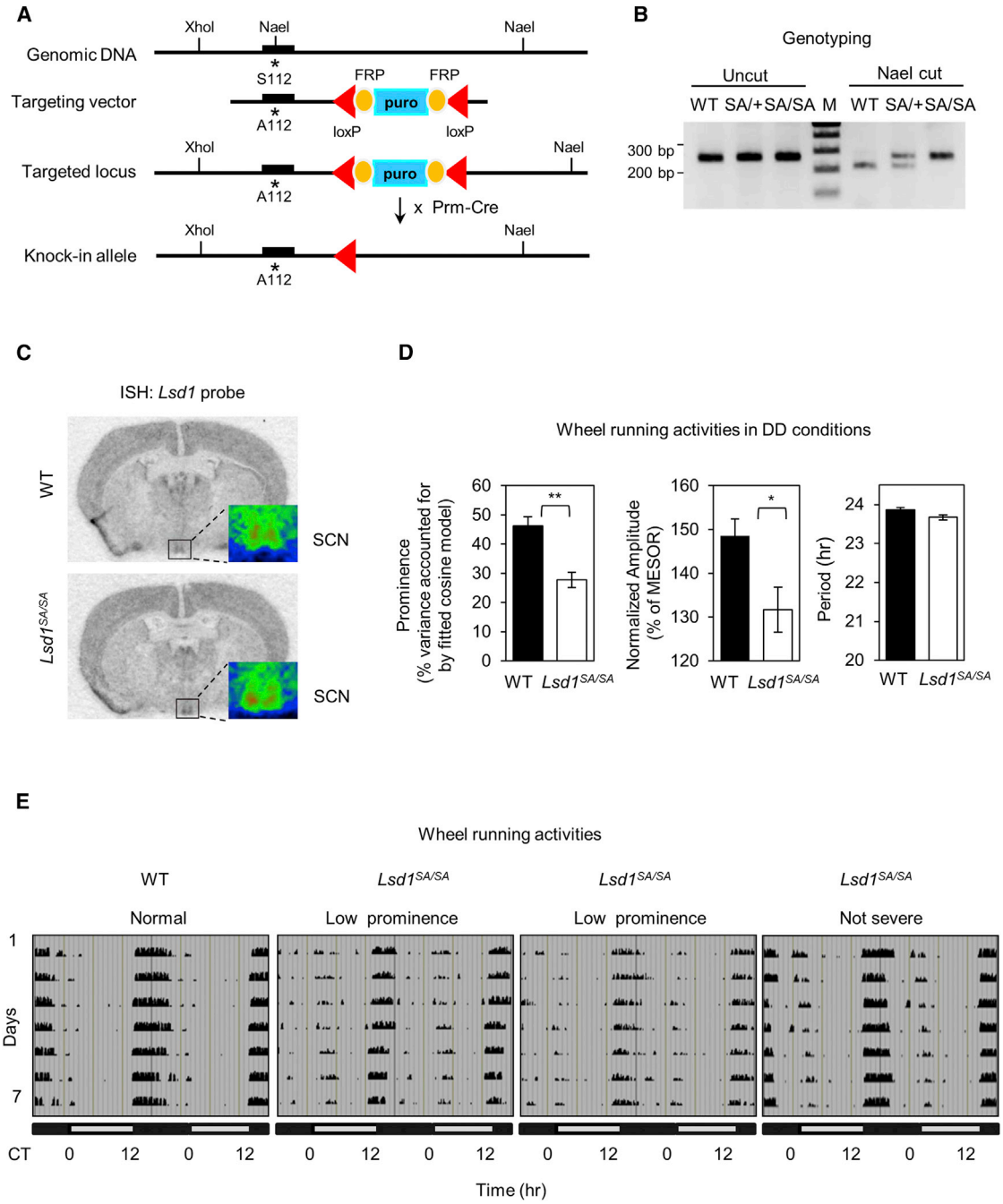


Figure 3. *Lsd1*$^{SA/SA}$ Knockin Mice Exhibit a Defect in Circadian Rhythm

(A) Strategy for generation of *Lsd1*$^{SA/SA}$ knockin mice. Site-directed mutagenesis introduced serine to alanine substitutions, which disrupted the NaeI site.

(B) Genotyping of WT, hetero, or *Lsd1*$^{SA/SA}$ mice. WT allele, but not *Lsd1*$^{SA/SA}$ allele, was digested by Nae1 restriction enzyme.

(C) In situ hybridization (ISH) analysis of *Lsd1* was performed in SCN tissues of WT and *Lsd1*$^{SA/SA}$ mice.

(D) Prominence of the rhythms, normalized amplitude, and period lengths of WT and *Lsd1*$^{SA/SA}$ mice. Prominence (or robustness) of a rhythm refers to the strength and endurance of a rhythm as a measure of the signal-to-noise ratio. To quantify rhythm prominence, the percentage of the variance accounted for by the fitted cosine model, which corresponds to the coefficient R^2 in regression analysis, was obtained after each cosinor analysis. Cosinor analysis was performed on daily rhythm of wheel-running activities under DD conditions. All data are expressed as mean ± SEM (n = 13 for WT, n = 15 for *Lsd1*$^{SA/SA}$, $^{**}p < 0.01$, $^{*}p < 0.05$; MESOR, midline-estimating statistic of a rhythm).

(E) Representative double-plotted actograms of WT and *Lsd1*$^{SA/SA}$ knockin mice. Each horizontal line represents 48 hr. The second 24 hr period is plotted to the right and below the first. Vertical bars represent periods of wheel-running activity. Animals were initially housed in a 12L:12D light-dark cycle for 14 days and were then transferred to constant darkness.

See also Table S2.

Mendelian distribution (data not shown). *Lsd1*$^{SA/SA}$ mice have been backcrossed to a C57BL/6 background for at least seven generations.

We first checked *Lsd1* expression in the SCN by in situ hybridization. WT and *Lsd1*$^{SA/SA}$ mice exhibited comparable *Lsd1* mRNA levels in the SCN (Figure 3C). In order to examine whether LSD1 phosphorylation influences circadian behavior of mice, we compared locomotor activity rhythms of WT and *Lsd1*$^{SA/SA}$ mice in light-dark (LD) or constant darkness (DD) conditions. In the LD photoperiod, the diurnal rhythmicity of locomotor behavior and distance moved per day were the same in the two genotypes (data not shown). However, under DD conditions, *Lsd1*$^{SA/SA}$ mice showed significantly less prominent rhythms with reduced amplitudes than those of the WT mice, although their free-running periods and acrophases were indistinguishable from those of the WT mice (Figure 3D; Table S2). The impaired prominence and reduced amplitude of the rhythm in the *Lsd1*$^{SA/SA}$ mice suggest their instability in maintaining the overt rhythm. Representative actograms showing the difference between WT and *Lsd1*$^{SA/SA}$ mice in their wheel-running activities under DD conditions are shown in Figure 3E.

Lsd1$^{SA/SA}$ Knockin Mice Show Attenuated Rhythmic Expression of Core Clock Genes in SCN

A distinct oscillation pattern of LSD1 phosphorylation was observed in SCN tissue of WT mice. The level of phosphorylated LSD1 in WT mice was higher during late daytime and early nighttime when CLOCK:BMAL1-mediated transcription is active; they are high between CT8 and CT12, peaked at CT8, and decreased thereafter (Figure 4A). However, the oscillation of LSD1 phosphorylation was largely absent in SCN of *Lsd1*$^{SA/SA}$ mice (Figure 4A). In addition, we found overlapping phosphorylation patterns of LSD1 and PKCα over a CT course, indicating in vivo correlation of LSD1 phosphorylation with PKCα activation in SCN, as in the case of liver tissues (Figure 2A). Further, we measured the PER2 protein profile over the course of a day in the SCN of WT and *Lsd1*$^{SA/SA}$ mice. Circadian changes in PER2 protein expression observed in the SCN of WT mice were almost abrogated in *Lsd1*$^{SA/SA}$ mice (Figure 4B).

We then compared temporal mRNA expression patterns of canonical clock genes, exemplified by *Per2*, *Rev-erbα*, *Cry1*, *Cry2*, and *Bmal1*, at different circadian times in the SCN from WT and *Lsd1*$^{SA/SA}$ mice. The cyclic accumulation of *Per2*, *Rev-erbα*, *Cry1*, and *Cry2* mRNAs in the SCN from *Lsd1*$^{SA/SA}$ mice was significantly attenuated compared to that from WT mice (Figure 4C). Compared to *Per2*, *Rev-erbα*, *Cry1*, and *Cry2*, which have functional *E-box* at the promoter, mRNA level of *Bmal1* under the control of *RORE*-mediated transcription (Mukherji et al., 2013) was not changed (Figure 4C). These data indicate that *E-box*-mediated periodic gene transcription is specifically attenuated in *Lsd1*$^{SA/SA}$ mice. Further, constant mRNA levels of *Lsd1* over the CT time course in both WT and *Lsd1*$^{SA/SA}$ mice (Figure 4C) supported constant expression levels of total LSD1 proteins throughout the CT time course as shown in Figure 4A.

Weakened circadian rhythmicity was further demonstrated in the cultured *Lsd1*$^{SA/SA}$ SCN explants. For real-time monitoring of SCN-autonomous rhythms, we produced *Lsd1*$^{SA/SA}$ mice expressing the luciferase (Luc) reporter fused to the *Per2* coding sequence (*Per2*^{Luc}) by cross-breeding the *Lsd1*$^{SA/SA}$ mice with *Per2*^{Luc} knockin mice (Yoo et al., 2004). We then measured *Per2*^{Luc} rhythms in SCN explants. Compared to the results from the WT controls, the SCN explant cultures from *Lsd1*$^{SA/SA}$ mice showed rhythms with lower amplitude, despite similar period lengths in the two genotypes (Figure 4D).

LSD1 Phosphorylation Is Required for Recruitment of CLOCK:BMAL1 Heterodimers to Target Gene Promoters

The finding of common phenotype of *Lsd1*$^{SA/SA}$ mice such as deregulation of circadian gene expression and impaired circadian behavior with that of *Bmal1* or *Clock*-deficient mice showing severe changes in circadian behavior and damped expression of circadian genes (Bunger et al., 2000; Debruyne et al., 2006) led us to further examine whether LSD1 functions as a molecular component of the circadian clockwork by regulating *E-box*-mediated transcription as a coactivator. Because recruitment of CLOCK:BMAL1 heterodimers to *E-box* elements and subsequent transcriptional activation are required for the circadian cycling of the molecular clock (Ripperger and Schibler, 2006), we performed experiments to ascertain the involvement of LSD1 phosphorylation in CLOCK:BMAL1-mediated transcription. Introduction of LSD1 WT increased CLOCK:BMAL1-induced luciferase activity with a reporter containing two functional *E-box* elements, whereas LSD1 SA mutant showed reduced luciferase activity compared to LSD1 WT (Figure 5A). The effect of LSD1 on CLOCK:BMAL1-mediated transcription was abolished in a mutant reporter lacking functional *E-box* elements by deletion of core nucleotides (CACGTG → CACTG) (Figure 5A). Consistent with *E-box*-mediated transcriptional activation function, LSD1 WT, but not SA mutant, increased *Per2* promoter-luciferase activity containing functional *E-box* elements (Figure 5B). LSD1 K661A mutant that has impaired enzymatic activity exhibited luciferase activity comparable to LSD1 WT (Figure 5B), suggesting the possibility that catalytic activity of LSD1 is not critical to the *E-box*-mediated transcriptional activation.

To eliminate the possibility that the failure of LSD1 SA mutant acting as a coactivator of CLOCK:BMAL1-mediated transcription is due to a change in cellular localization, we checked cellular localization of LSD1 WT and SA mutant. Nuclear localization of LSD1 in WT MEFs was not altered in *Lsd1*$^{SA/SA}$ MEFs, indicating that LSD1 phosphorylation does not affect cellular localization of LSD1 (Figure 5C). Next, we examined the possibility whether PKCα-dependent phosphorylation of LSD1 affects its intrinsic histone demethylase (HDM) activity, although S112 phosphorylation site is not located within amine oxidase region of LSD1, which is crucial for enzymatic activity (Forneris et al., 2007; Yang et al., 2006a). We performed in vitro HDM activity assays with purified enzymes (Figure 5D) or immunoprecipitated materials (Figure S1B) and found that LSD1 WT and SA mutant exhibited comparable HDM activity on H3K4me2. Immunofluorescence assay with LSD1 WT, SA mutant, and KA mutant supported the in vitro HDM activity assay results (Figure 5E). Together, these data indicate that PKCα-dependent phosphorylation of LSD1 does not alter its intrinsic HDM activity.

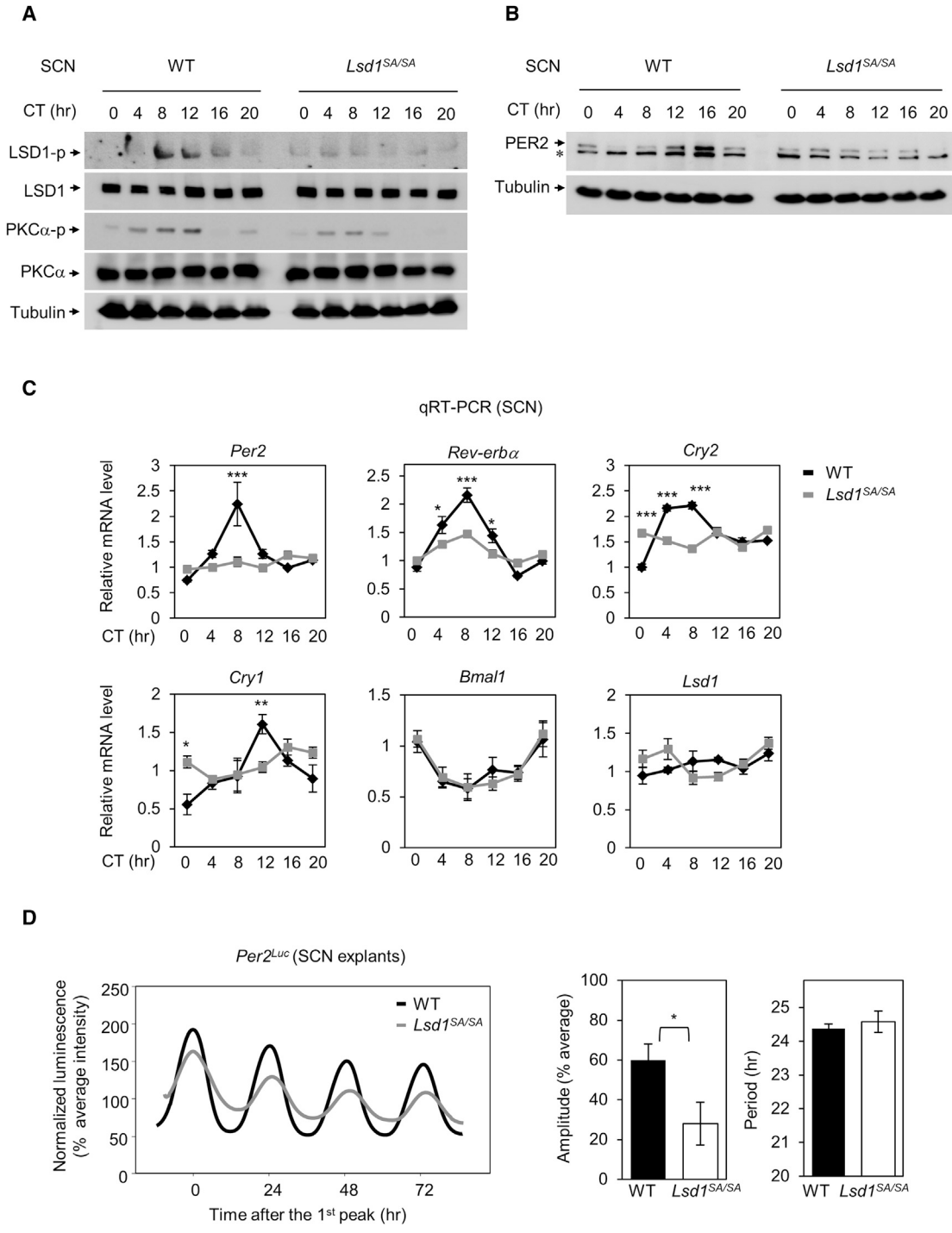
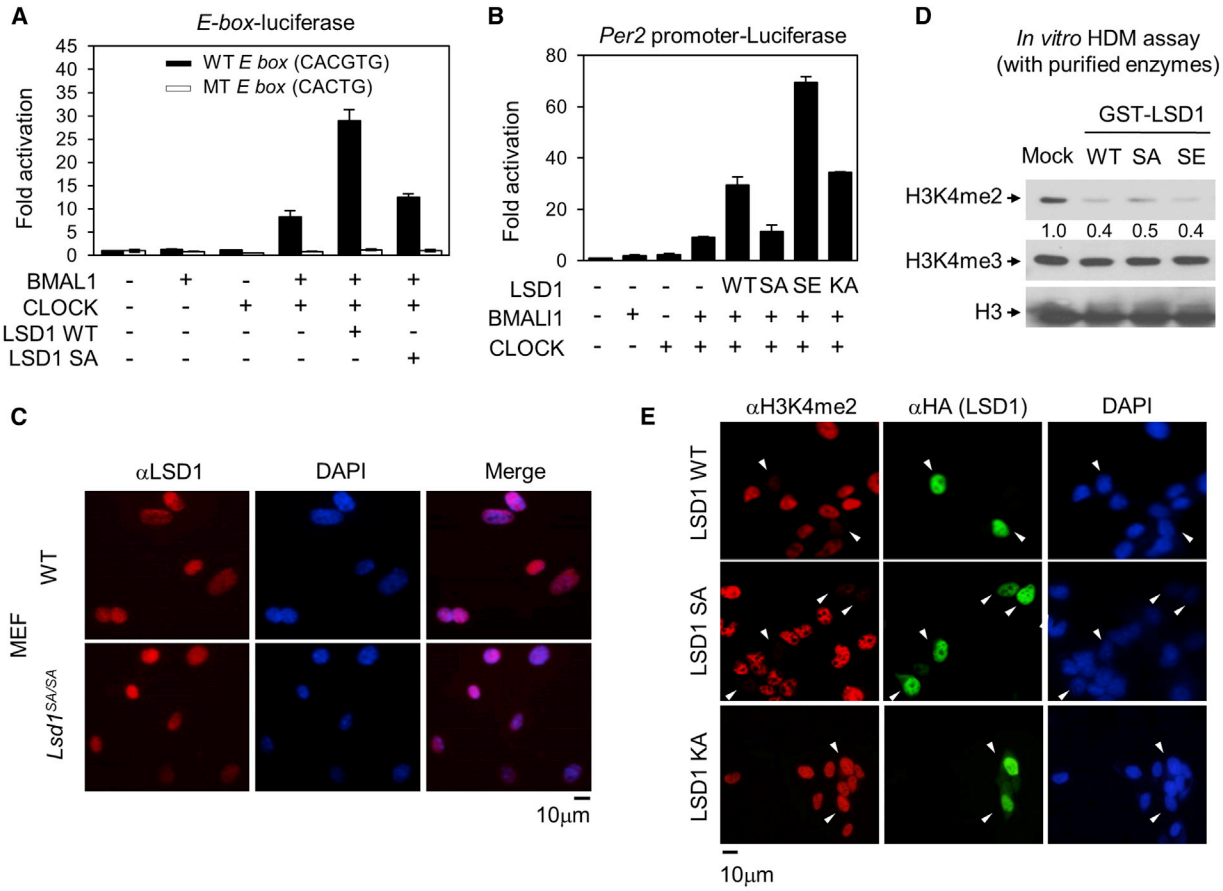


Figure 4. *Lsd1*$^{SA/SA}$ Knockin Mice Show Attenuated Rhythmic Expression of Core Clock Genes

(A and B) Immunoblot analysis was performed in mouse SCN extracts. Mouse SCNs were collected in a DD cycle, and SCNs of three mice at each CT time were pooled for immunoblot analysis with anti-LSD1-p, LSD1, PKCα-p, and PKCα antibodies (A) or anti-PER2 antibody (B). *Nonspecific band.

(C) Quantitative RT-PCR analysis of *Per2, Rev-erbα*, *Cry2, Cry1*, *Bmal1*, and *Lsd1* was conducted using SCN tissue extracts obtained from WT or *Lsd1*$^{SA/SA}$ mice 4 hr intervals. Average normalized values were plotted at various time points. All data are presented as mean ± SEM (n = 3–4 at each point, *p < 0.05, **p < 0.01, ***p < 0.001).

(D) LSD1 phosphorylation is required for sustained *Per2*^{Luc} rhythms in SCN explant cultures. Luminescence emitted from SCN explant cultures was continuously monitored at 10 min intervals. Black line shows an average profile of Per2::Luciferase oscillation in WT, and gray line shows average profile in *Lsd1*$^{SA/SA}$. Graph depicts the luciferase amplitude and period lengths. Data are expressed as mean ± SEM (n = 5 for WT, n = 4 for *Lsd1*$^{SA/SA}$, *p < 0.05).



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In parallel, mRNA level of *Per2*, a CLOCK:BMAL1 target gene, was monitored in *Lsd1*$^{-/-}$ MEFs reconstituted with LSD1 WT, SA mutant, or SE mutant, which mimics constitutive phosphorylation of LSD1. Compared to LSD1 WT or SE mutant, SA mutant significantly attenuated *Per2* mRNA levels (Figure S1C). Quantitative RT-PCR analysis confirmed that *Per2* mRNA level in *Lsd1*$^{SA/SA}$ MEFs was lower than that in WT MEFs (Figure S1D). Consistent with reduction of *Per2* mRNA level in *Lsd1*$^{SA/SA}$ MEFs, chromatin immunoprecipitation (ChIP) assay showed that LSD1 recruitment to the *Per2* promoter was decreased in *Lsd1*$^{SA/SA}$ MEFs (Figure S1E). To examine whether recruitment of LSD1 to *Per2* promoter is dependent on CLOCK:BMAL1 transcription factors, we performed ChIP assay in *Bmal1*$^{-/-}$ MEFs. LSD1 failed to be recruited to *Per2* promoter in *Bmal1*$^{-/-}$ MEFs (Figure S1F), indicating that LSD1 recruitment is mediated by CLOCK:BMAL1 heterodimers.

To examine whether recruitment of CLOCK:BMAL1 is affected by LSD1 phosphorylation, we performed ChIP assays on *Per2* and *Dbp* promoters in WT and *Lsd1*$^{SA/SA}$ mouse livers. In *Lsd1*$^{SA/SA}$ mouse livers, CLOCK:BMAL1 recruitment at CT8–12 was attenuated on *Per2* and *Dbp* promoters, but histone H3K4me2 and H3K9me2 levels were not significantly changed compared to those in WT mouse livers (Figures 5F, 5G, and S1G). Consistent with previous report (Koike et al., 2012), histone acetylation level of H3K9 was well overlapped with recruitment of CLOCK:BMAL1 transcription factors in WT mouse livers, but H3K9 acetylation level was attenuated in *Lsd1*$^{SA/SA}$ mouse livers (Figures 5F, 5G, and S1G). Our findings indicate that LSD1 phosphorylation plays a central role in CLOCK:BMAL1-dependent transcription of core clock genes by facilitating both recruitment of CLOCK:BMAL1 to the target promoters and LSD1 binding to CLOCK:BMAL1 proteins, thereby producing a normal behavioral rhythm under free-running conditions. The LSD1 function for the activation of CLOCK:BMAL1-dependent transcription appears to be independent of its enzymatic activity.

Lsd1$^{SA/SA}$ Knockin Mice Show Impaired *Per1* Induction by Photic Stimuli

To keep pace with the light-dark cycle, the SCN master clock can be reset by light stimuli (Bae et al., 2001). A light pulse perceived during early night delays the phase of circadian pacemaker, whereas a pulse applied in late night advances it. Because the PKC pathway mediates photic entrainment of the SCN central clock (Jakubcakova et al., 2007; Shim et al., 2007), we examined whether LSD1 phosphorylation is required for the phase resetting by photic signals. We checked whether a light pulse alters LSD1 phosphorylation levels at both CT14 and CT22 in SCN tissues. Immunoblot analysis revealed that LSD1 phosphorylation on S112 site was acutely elevated by a light pulse at both CT14 (Figure 6A) and CT22 (Figure 6B) with the PKCα induction. Given that *Per1* induction is required for light-induced phase resetting (Albrecht et al., 1997, 2001; Bae et al., 2001), it was noteworthy that lack of LSD1 phosphorylation in the *Lsd1*$^{SA/SA}$ mice significantly attenuated the induction of *Per1* mRNA and protein in the SCN by a light pulse (Figures 6A–6D; $^{**}p < 0.01$, $^{*}p < 0.05$).

Because activation of the PKC signaling pathway has been shown to function as one of the phase resetting signals in the molecular clock in cultured fibroblasts (Akashi and Nishida, 2000; Shim et al., 2007), we treated MEFs with PMA. Consistent with our SCN data, induction of *Per1* was attenuated in *Lsd1*$^{SA/SA}$ MEFs compared to WT MEFs (Figure 6E). Further, we performed a ChIP assay to monitor whether LSD1 is recruited to *Per1* promoter for transcriptional activation. A light pulse at CT14 in SCN tissues induced recruitment of LSD1 on the *Per1* promoter (Figure 6F). Together, these data indicate that LSD1 phosphorylation is crucial for the phase resetting by photic signals.

Lsd1$^{SA/SA}$ Knockin Mice Show Impaired Behavioral Adaptation to Photic Stimuli

In accordance with the attenuated induction of *Per1* expression by photic stimuli in *Lsd1*$^{SA/SA}$ mice, *Lsd1*$^{SA/SA}$ mice showed impaired behavioral adaptation to photic stimuli, as shown by wheel-running activity (Figures 7A and 7B). *Lsd1*$^{SA/SA}$ mice exhibited an abnormal behavioral response to short nocturnal light pulses, which can reset the phase of the endogenous clock. Quantitative differences were observed at the extent of phase delays and advances. Exposure to a light pulse at CT14 caused a phase delay in WT animals (-113.91 ± 5.49 min), whereas *Lsd1*$^{SA/SA}$ mice showed an impaired delay (-66.16 ± 17.63 min, $^{*}p < 0.05$) (Figure 7A). Comparison of the phase of rhythmicity after light pulse revealed that the phase shifts in the WT and *Lsd1*$^{SA/SA}$ mice were statistically significant ($^{*}p < 0.05$). A light pulse at CT22 in the same animals led to comparable phase advances in WT mice (34.94 ± 8.68 min), but not in the *Lsd1*$^{SA/SA}$ mice (-28.52 ± 24.07 min, $^{*}p < 0.05$) (Figure 7B). These findings collectively demonstrate that LSD1 phosphorylation mediates the phase resetting mechanism of the central circadian clock.

Figure 5. LSD1 Phosphorylation Is Required for Recruitment of CLOCK:BMAL1 Heterodimers to Target Gene Promoters

(A) Two E-boxes were fused to the SV40 minimal promoter-luciferase reporter. HEK293T cells were cotransfected with WT or mutant E-box-luciferase reporter, BMAL1, CLOCK, LSD1 WT, or SA mutant, as indicated. Data are expressed as mean ± SD.

(B) Luciferase reporter assay was performed with *Per2* promoter-luciferase reporter as in (A). Data are expressed as mean ± SD.

(C) Immunostaining of WT or *Lsd1*$^{SA/SA}$ MEFs with antibodies against LSD1 (red). The nucleus was stained with DAPI (blue).

(D) In vitro HDM assays using purified GST-LSD1 WT, SA, or SE proteins were performed, and histone H3K4me2 and H3K4me3 levels were detected along with histone H3 levels by immunoblot analysis.

(E) Immunofluorescence assay shows that LSD1 SA mutant possesses comparable demethylase activity on H3K4me2 to that of LSD1 WT. LSD1 KA mutant, which has impaired histone demethylase activity, was used as a negative control.

(F and G) ChIP assays were performed on the *Per2* (F) and *Dbp* (G) promoters in WT and *Lsd1*$^{SA/SA}$ mouse livers over a circadian time course. Mouse livers were collected for 4 hr hr intervals in a DD cycle at indicated CT time. Data are expressed as mean ± SEM.

See also Figure S1.

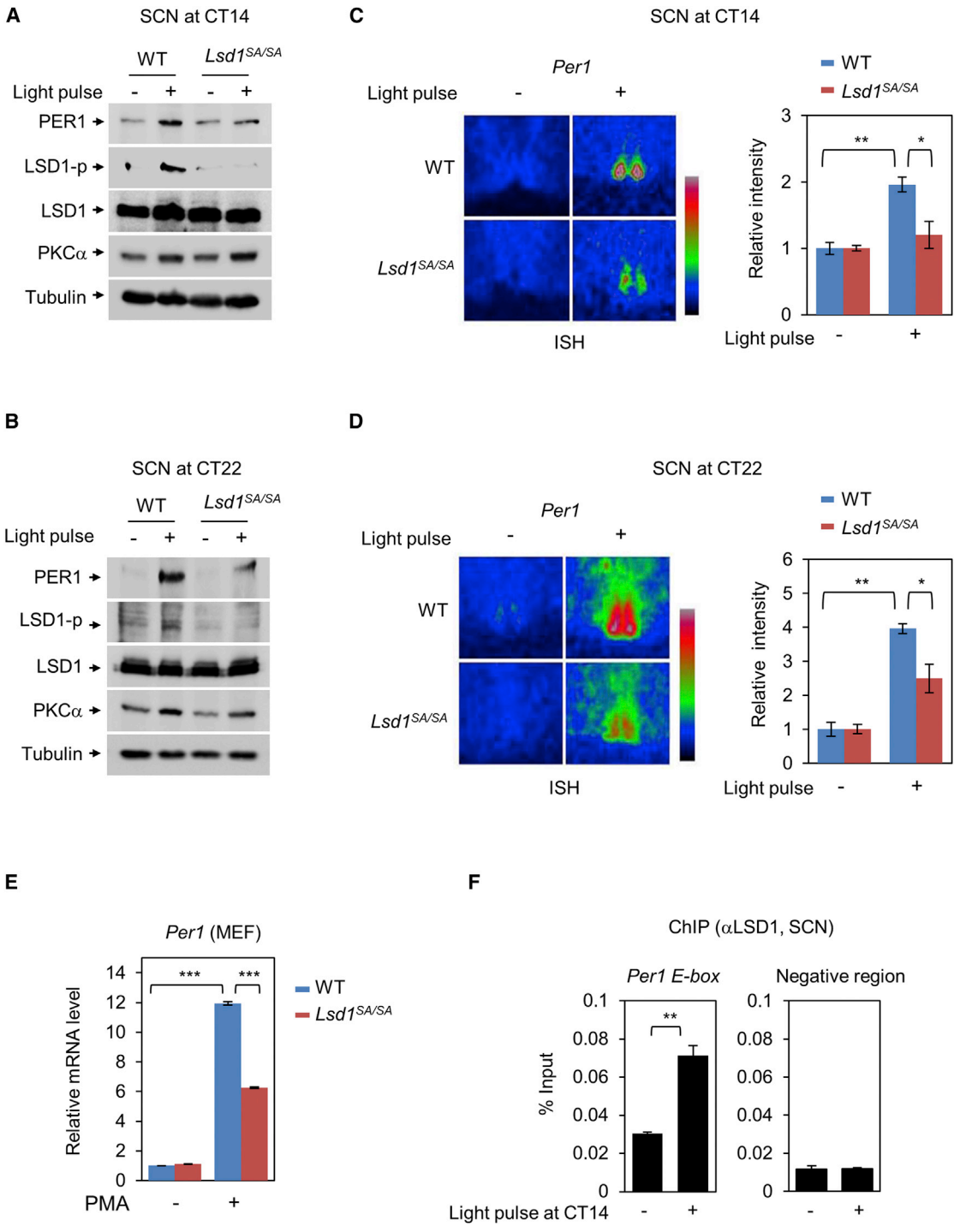


Figure 6. *Lsd1*$^{SA/SA}$ Knockin Mice Show Impaired *Per1* Induction by Photic Stimuli

(A and B) Immunoblot analysis was performed with mouse SCN extracts. WT and *Lsd1*$^{SA/SA}$ mice were exposed to a 30 min light pulse at CT14 (A) or CT22 (B) and sacrificed after 30 min. SCNs of three mice at each time point were pooled for immunoblot analysis.

(C and D) In situ hybridization analysis of *Per1* was performed at SCN tissues. WT and *Lsd1*$^{SA/SA}$ mice were exposed to a 30 min light pulse at CT14 (C) or CT22 (D) and sacrificed after 30 min. Bar plots show quantification of mRNA induction by densitometric analysis of X-ray film contact autoradiographs. The intensity of the signal of WT controls not exposed to light at CT15 was set as 1. All data are expressed as mean ± SEM (n = 3–4 per condition and genotype, $^{**}p < 0.01$, $^{*}p < 0.05$).

(legend continued on next page)

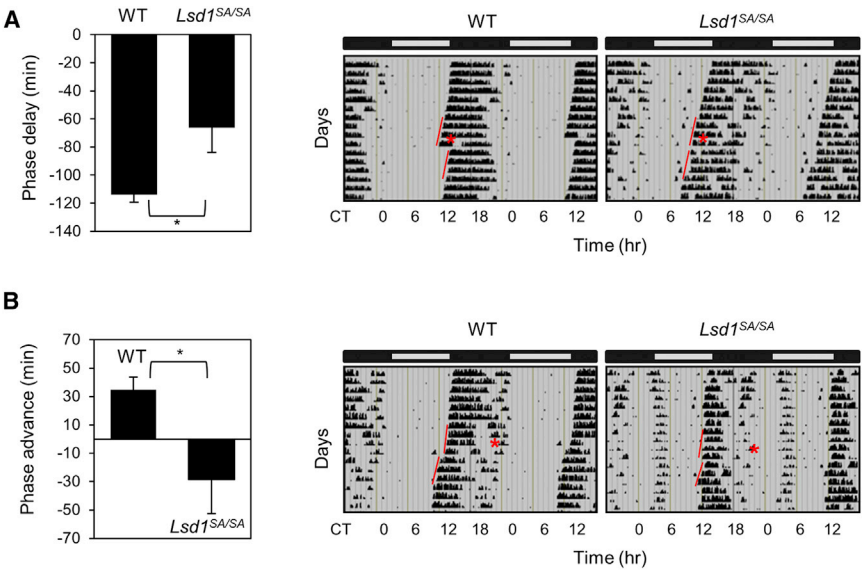
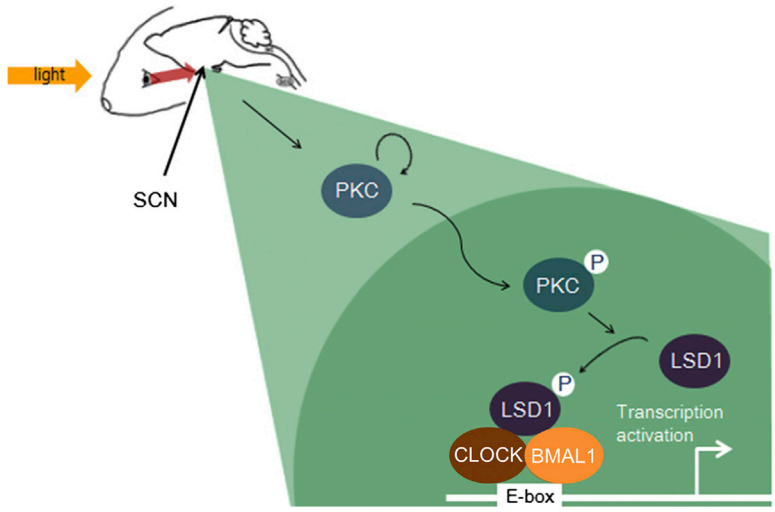


Figure 7. *Lsd1*$^{SA/SA}$ Knockin Mice Show Impaired Behavioral Adaptation to Photic Stimuli

(A and B) WT and *Lsd1*$^{SA/SA}$ mice exposed to a 30 min light pulse (red asterisk) either in the early (CT14) or late (CT22) subjective night. All data are presented as mean ± SEM (n = 6–8 per condition and genotype, *p < 0.05). Right panels show representative double-plotted actograms of WT or *Lsd1*$^{SA/SA}$ mice. Each horizontal line represents 48 hr. The second 24 hr period is plotted to the right and below the first. Vertical bars represent periods of wheel-running activity. Animals were initially housed in a 12 hr light:12 hr dark cycle for at least 2 weeks and were then transferred to constant darkness.

(C) Schematic model of PKCα-induced LSD1 phosphorylation playing an important role in oscillation of the molecular circadian clock and phase resetting of circadian clock. LSD1 is phosphorylated by PKCα in a circadian manner, and the phosphorylated LSD1 forms a complex with CLOCK:BMAL1 heterodimers to facilitate *E-box*-mediated transcriptional activation.



DISCUSSION

Our *Lsd1*$^{SA/SA}$ mouse model is a knockin mouse model of a phosphorylation-deficient histone demethylase that demonstrates in vivo functions of phosphorylation-modulated LSD1. Phenotypic analyses of *Lsd1*$^{SA/SA}$ knockin mice showed a defect in circadian rhythmicity, impaired phase resetting of the circadian clock, and impaired behavioral adaptation to photic stimuli. LSD1 is phosphorylated by PKCα in a circadian manner, and the phosphorylated LSD1 forms a complex with CLOCK:BMAL1 heterodimers to facilitate *E-box*-mediated transcriptional activation (Figure 7C). Prior studies have highlighted the role for histone methyltransferases Mll1 (Katada and Sassone-Corsi, 2010), PRMT5 (Sanchez et al., 2010), and Mll3 (Valekunja et al., 2013) in regulating circadian transcription, but have not addressed the question of how the recruitment and activity of these enzymes are regulated by signaling events to result in the cyclical nature of action. JARID1a histone demethylase has been shown to increase histone acetylation by inhibiting histone deacetylase activity and thus enhance transcriptional activation of target genes by CLOCK:BMAL1 (DiTacchio et al., 2011). It is interesting that the catalytic activity of LSD1 is not required for the phosphorylation-induced coactivator function of LSD1 at CLOCK:BMAL1 target genes, an observation similar to that of the JARID1a. Future studies will elucidate how LSD1 acts as a coactivator independent of its catalytic activity and how signal transduction cascade target chromatin modifiers including LSD1 to control dynamic circadian gene expression programs.

Using *Lsd1*$^{SA/SA}$ knockin mouse model, we demonstrate that phosphorylation of LSD1 by PKCα, in response to acute photic stimulus as well as in a circadian manner, is evidently required for both normal cycling and the photic entrainment of the

(E) Quantitative RT-PCR analysis of *Per1* was conducted using LSD1 WT and *Lsd1*$^{SA/SA}$ MEF. MEFs were serum starved for 24 hr and treated with 1 μM PMA for 30 min. Data are expressed as mean ± SEM (n = 3 each, ***p < 0.001).

(F) ChIP assays were performed on the *Per1* promoter using WT and *Lsd1*$^{SA/SA}$ SCN tissue. WT and *Lsd1*$^{SA/SA}$ mice were exposed to a 30 min light pulse at CT14 and sacrificed after 30 min. SCNs of three mice at each time point were pooled for ChIP assay. Relative enrichment of LSD1 on the *Per1* promoter was analyzed by quantitative PCR. Data are expressed as mean ± SEM (n = 3, **p < 0.01).

circadian clock. PKC signaling mediates various neuropeptidergic inputs to the SCN, thereby playing a pivotal role in the entrainment of the circadian clock presumably by directly phosphorylating canonical clock proteins such as CLOCK (Shim et al., 2007). However, it has not yet been proved that phosphorylation of canonical clock proteins is sufficient for PKC-driven phase resetting. Our findings using the *Lsd1*$^{SA/SA}$ mouse model strongly support that LSD1, a direct substrate of PKCα, is crucial for integrating the clock resetting mechanism in phosphorylation-dependent manner in vivo.

It was quite unexpected that PKCα-induced LSD1 phosphorylation plays an important role in the 24 hr oscillation of the molecular circadian clock by modulating efficient recruitment of CLOCK:BMAL1 heterodimers to the promoter region of clock-controlled genes such as *Per2*. Enhanced activity of PKCα by RACK1 has been shown to phosphorylate BMAL1, which, in turn, leads to changes in the period of the clock (Robles et al., 2010). Here, we postulate LSD1 to be a key component of an activating complex in the positive feedback loops of the molecular clockwork and demonstrate that PKCα activity stimulates CLOCK:BMAL1 transcription activity through LSD1 phosphorylation. The overall data suggest that PKCα activity is modulated by RACK1, which is mainly associated with CLOCK:BMAL1 during its repression phase (Robles et al., 2010), whereas LSD1 operates only during the activation phase (i.e., CT4-12) as a strong binder of PKCα. We speculate that PKCα can bidirectionally regulate transcriptional activity of CLOCK:BMAL1 heterodimer in a phase-dependent manner via employing alternative pathways.

In this regard, it should be noted that cytosolic rhythms manifested by daily oscillations of intracellular second messengers, such as cytosolic Ca^{2+} and adenosine 3′,5′-monophosphate (cAMP), have been recently found to integrate with the nuclear rhythm generated by the transcription/translation feedback loops in the SCN pacemaker neurons (Brancaccio et al., 2013; O'Neill et al., 2008). Furthermore, the Gq-Ca^{2+} system, a major upstream regulator of PKC signaling, is strongly involved in controlling synchrony of the SCN pacemakers to produce overt rhythms (Brancaccio et al., 2013). Therefore, it is tempting to speculate that PKCα-LSD1 signaling axis functions as an orchestrating link between nuclear and cytosolic rhythms both to maintain robust circadian rhythms in a variety of cellular processes and to adjust the phases in response to extracellular stimuli in the SCN pacemaker neurons and other peripheral systems. Together, our data demonstrate that LSD1 is a key component of the molecular circadian oscillator, which functions in facilitating circadian rhythmicity and phase resetting of the circadian clock.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J mice at 8–10 weeks of age were used in the experiments. Mice were housed under controlled conditions of temperature (22°C –23°C) and light (12 hr light:12 hr dark, lights switched on at 8:00 AM). Food and water were available ad libitum. For dark-dark conditions, mice were kept in constant darkness for the indicated duration from the lights-off time. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University.

Generation of *Lsd1*$^{SA/SA}$ Knockin Mice

A targeting vector containing replacement of serine 112 to alanine was electroporated into ES cells, and positive clones with homologous recombination at the *Lsd1* locus were selected for electroporation with a plasmid expressing protamine-cre recombinase to remove the Puro^r cassette. A heterozygous *Lsd1*$^{SA/+}$ ES clone was selected and injected into mouse blastocysts, yielding chimeric mice, which transmitted the mutant allele. The C57BL/6 background F7 generation was genotyped by PCR analysis of tail DNAs using allele-specific primers, and the mutant mice were confirmed by both NaeI digestion and sequencing of PCR products. Genotyping primers were as follows: forward 5′-CATGGAGACCGGAATAGCCGAG-3′ and reverse 5′-TACACAACC CAGAAGCCGTC-3′.

SCN-ChIP Assays

Whole-brain samples were quickly harvested at the indicated time points. To obtain the SCN-enriched sample, we cut anterior hypothalamus and punched-out biopsies of the SCN region from the anterior hypothalamus. It was quickly washed with PBS and crosslinked in 1% formaldehyde for 10 min, followed by quenching with 0.125 M glycine solution for 5 min and washes with ice-cold PBS two times. Chromatin fragmentation was performed by sonication in ChIP lysis buffer (50 mM Tris-HCl [pH 8.1], 1% SDS, 10 mM EDTA [pH 7.6], protease inhibitor cocktail). Proteins were immunoprecipitated in ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], and protease inhibitor cocktail). Crosslinking was reversed overnight at 65°C in elution buffer (1% SDS, 0.1 M $NaHCO_3$), and DNA was purified with a QIAquick Gel extraction Kit (QIAGEN). Precipitated DNA was analyzed by quantitative PCR. For real-time quantitative PCR analysis, 2 μl from 50 μl DNA extractions was used. The following primers were used (Busino et al., 2007): *Per2* (TSS −150 bp∼+1 bp *E*-box region) forward: 5′-AGCAG CATCTTCATTGAGGAACCCGGG-3′, *Per2* (TSS −150 bp∼+1 bp *E*-box region) reverse: 5′-CTCCGCTGTCACATAGTGGAAAACGTGAC-3′, *Per1* (TSS −4,081 bp∼−3,969 bp *E*-box region) forward: 5′-AGCCAGCCTG CACGTGTTCC-3′, *Per1* (TSS −4081 bp∼−3,969 bp *E*-box region) reverse: 5′-CAGAGACAACCCCGCCCTGC-3′, *Dbp* (TSS −461 bp∼−390 bp *E*-box region) forward: 5′-ACACCCGCATCCGATAGC-3′, *Dbp* (TSS −461 bp∼−390 bp *E*-box region) reverse: 5′-CCACTTCGGGCCAATGAG-3′, negative region forward: 5′-CCACACGGTACTCAGCGGGC-3′, and negative region reverse: 5′-GGGTCACTGCGAGCCTTGCC-3′.

Statistical Analysis

Data were analyzed by Student's t tests for group differences, by one-way ANOVA for time differences and group differences separately, and by two-way ANOVA for time and group differences. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.01.028.

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REFERENCES

Akashi, M., and Nishida, E. (2000). Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev. *14*, 645–649.

Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell *91*, 1055–1064.

Albrecht, U., Zheng, B., Larkin, D., Sun, Z.S., and Lee, C.C. (2001). MPer1 and mper2 are essential for normal resetting of the circadian clock. J. Biol. Rhythms *16*, 100–104.

Asher, G., Gatfield, D., Stratmann, M., Reinke, H., Dibner, C., Kreppel, F., Mostoslavsky, R., Alt, F.W., and Schibler, U. (2008). SIRT1 regulates circadian clock gene expression through PER2 deacetylation. Cell *134*, 317–328.

Bae, K., Jin, X., Maywood, E.S., Hastings, M.H., Reppert, S.M., and Weaver, D.R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525–536.

Brancaccio, M., Maywood, E.S., Chesham, J.E., Loudon, A.S., and Hastings, M.H. (2013). A Gq-Ca Axis Controls Circuit-Level Encoding of Circadian Time in the Suprachiasmatic Nucleus. Neuron *78*, 1–15.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S., and Bradfield, C.A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009–1017.

Busino, L., Bassermann, F., Maiolica, A., Lee, C., Nolan, P.M., Godinho, S.I., Draetta, G.F., and Pagano, M. (2007). SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. Science *316*, 900–904.

Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J.J., and Sassone-Corsi, P. (2005). Circadian clock control by SUMOylation of BMAL1. Science *309*, 1390–1394.

Cheung, P., Allis, C.D., and Sassone-Corsi, P. (2000). Signaling to chromatin through histone modifications. Cell *103*, 263–271.

Debruyne, J.P., Noton, E., Lambert, C.M., Maywood, E.S., Weaver, D.R., and Reppert, S.M. (2006). A clock shock: mouse CLOCK is not required for circadian oscillator function. Neuron *50*, 465–477.

DiTacchio, L., Le, H.D., Vollmers, C., Hatori, M., Witcher, M., Secombe, J., and Panda, S. (2011). Histone lysine demethylase JARID1a activates CLOCK-BMAL1 and influences the circadian clock. Science *333*, 1881–1885.

Doi, M., Hirayama, J., and Sassone-Corsi, P. (2006). Circadian regulator CLOCK is a histone acetyltransferase. Cell *125*, 497–508.

Etchegaray, J.P., Lee, C., Wade, P.A., and Reppert, S.M. (2003). Rhythmic histone acetylation underlies transcription in the mammalian circadian clock. Nature *421*, 177–182.

Etchegaray, J.P., Yang, X., DeBruyne, J.P., Peters, A.H., Weaver, D.R., Jenuwein, T., and Reppert, S.M. (2006). The polycomb group protein EZH2 is required for mammalian circadian clock function. J. Biol. Chem. *281*, 21209–21215.

Forneris, F., Binda, C., Adamo, A., Battaglioli, E., and Mattevi, A. (2007). Structural basis of LSD1-CoREST selectivity in histone H3 recognition. J. Biol. Chem. *282*, 20070–20074.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Huang, J., Sengupta, R., Espejo, A.B., Lee, M.G., Dorsey, J.A., Richter, M., Opravil, S., Shiekhattar, R., Bedford, M.T., Jenuwein, T., and Berger, S.L. (2007). p53 is regulated by the lysine demethylase LSD1. Nature *449*, 105–108.

Jakubcakova, V., Oster, H., Tamanini, F., Cadenas, C., Leitges, M., van der Horst, G.T., and Eichele, G. (2007). Light entrainment of the mammalian circadian clock by a PRKCA-dependent posttranslational mechanism. Neuron *54*, 831–843.

Katada, S., and Sassone-Corsi, P. (2010). The histone methyltransferase MLL1 permits the oscillation of circadian gene expression. Nat. Struct. Mol. Biol. *17*, 1414–1421.

Koike, N., Yoo, S.H., Huang, H.C., Kumar, V., Lee, C., Kim, T.K., and Takahashi, J.S. (2012). Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science *338*, 349–354.

Lee, M.G., Wynder, C., Cooch, N., and Shiekhattar, R. (2005). An essential role for CoREST in nucleosomal histone 3 lysine 4 demethylation. Nature *437*, 432–435.

Metzger, E., Wissmann, M., Yin, N., Müller, J.M., Schneider, R., Peters, A.H., Günther, T., Buettner, R., and Schüle, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature *437*, 436–439.

Mohawk, J.A., Green, C.B., and Takahashi, J.S. (2012). Central and peripheral circadian clocks in mammals. Annu. Rev. Neurosci. *35*, 445–462.

Mukherji, A., Kobiita, A., Ye, T., and Chambon, P. (2013). Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. Cell *153*, 812–827.

Mulligan, P., Yang, F., Di Stefano, L., Ji, J.Y., Ouyang, J., Nishikawa, J.L., Toiber, D., Kulkarni, M., Wang, Q., Najafi-Shoushtari, S.H., et al. (2011). A SIRT1-LSD1 corepressor complex regulates Notch target gene expression and development. Mol. Cell *42*, 689–699.

O'Neill, J.S., Maywood, E.S., Chesham, J.E., Takahashi, J.S., and Hastings, M.H. (2008). cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science *320*, 949–953.

Ripperger, J.A., and Schibler, U. (2006). Rhythmic CLOCK-BMAL1 binding to multiple E-box motifs drives circadian Dbp transcription and chromatin transitions. Nat. Genet. *38*, 369–374.

Robles, M.S., Boyault, C., Knutti, D., Padmanabhan, K., and Weitz, C.J. (2010). Identification of RACK1 and protein kinase Calpha as integral components of the mammalian circadian clock. Science *327*, 463–466.

Sanchez, S.E., Petrillo, E., Beckwith, E.J., Zhang, X., Rugnone, M.L., Hernando, C.E., Cuevas, J.C., Godoy Herz, M.A., Depetris-Chauvin, A., Simpson, C.G., et al. (2010). A methyl transferase links the circadian clock to the regulation of alternative splicing. Nature *468*, 112–116.

Schak, K.M., and Harrington, M.E. (1999). Protein kinase C inhibition and activation phase advances the hamster circadian clock. Brain Res. *840*, 158–161.

Shearman, L.P., Sriram, S., Weaver, D.R., Maywood, E.S., Chaves, I., Zheng, B., Kume, K., Lee, C.C., van der Horst, G.T., Hastings, M.H., and Reppert, S.M. (2000). Interacting molecular loops in the mammalian circadian clock. Science *288*, 1013–1019.

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A., and Shi, Y. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell *119*, 941–953.

Shim, H.S., Kim, H., Lee, J., Son, G.H., Cho, S., Oh, T.H., Kang, S.H., Seen, D.S., Lee, K.H., and Kim, K. (2007). Rapid activation of CLOCK by Ca2+-dependent protein kinase C mediates resetting of the mammalian circadian clock. EMBO Rep. *8*, 366–371.

Valekunja, U.K., Edgar, R.S., Oklejewicz, M., van der Horst, G.T., O'Neill, J.S., Tamanini, F., Turner, D.J., and Reddy, A.B. (2013). Histone methyltransferase MLL3 contributes to genome-scale circadian transcription. Proc. Natl. Acad. Sci. USA *110*, 1554–1559.

Van der Zee, E.A., and Bult, A. (1995). Distribution of AVP and Ca(2+)-dependent PKC-isozymes in the suprachiasmatic nucleus of the mouse and rabbit. Brain Res. *701*, 99–107.

Wang, J., Hevi, S., Kurash, J.K., Lei, H., Gay, F., Bajko, J., Su, H., Sun, W., Chang, H., Xu, G., et al. (2009). The lysine demethylase LSD1 (KDM1) is required for maintenance of global DNA methylation. Nat. Genet. *41*, 125–129.

Welsh, D.K., Takahashi, J.S., and Kay, S.A. (2010). Suprachiasmatic nucleus: cell autonomy and network properties. Annu. Rev. Physiol. *72*, 551–577.

Yang, M., Gocke, C.B., Luo, X., Borek, D., Tomchick, D.R., Machius, M., Otwinowski, Z., and Yu, H. (2006a). Structural basis for CoREST-dependent

demethylation of nucleosomes by the human LSD1 histone demethylase. Mol. Cell *23*, 377–387.

Yang, X., Downes, M., Yu, R.T., Bookout, A.L., He, W., Straume, M., Mangelsdorf, D.J., and Evans, R.M. (2006b). Nuclear receptor expression links the circadian clock to metabolism. Cell *126*, 801–810.

Yoo, S.H., Yamazaki, S., Lowrey, P.L., Shimomura, K., Ko, C.H., Buhr, E.D., Siepka, S.M., Hong, H.K., Oh, W.J., Yoo, O.J., et al. (2004). PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. USA *101*, 5339–5346.

Yoo, S.H., Mohawk, J.A., Siepka, S.M., Shan, Y., Huh, S.K., Hong, H.K., Kornblum, I., Kumar, V., Koike, N., Xu, M., et al. (2013). Competing E3 ubiquitin ligases govern circadian periodicity by degradation of CRY in nucleus and cytoplasm. Cell *152*, 1091–1105.