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CDK9 modulates circadian clock by attenuating REV-ERBα activity

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ABSTRACT

Circadian clock and cell cycle are vital cellular programs acting in a timely-regulated, cyclic manner. The two cellular oscillators are coupled in various ways to facilitate biological processes. Here we report CDK9, a kinase belongs to the CDK family in regulating cell cycle and RNA Pol II activity, can serve as a modulator for circadian clock. We identified CDK inhibitor LY2857785 potently blocked PER2:LUC expression in MEFs from a screen of 17 commonly-used CDK inhibitors. We further analyzed the possible targets of LY2857785 by siRNA approach, and confirmed CDK9 as the main effector. LY2857785 treatment, as well as *Cdk9* knock-down, led to lowered expression of *Bmal1* in accordance with elevated expression of *Rev-Erbα*. CDK9 associated with REV-ERBα thus attenuated REV-ERBα binding to the RORE for *Bmal1* suppression. To conform the circadian-modulating activity of CDK9 *in vivo*, we knocked down CDK9 in mice at the anterior hypothalamus covering the central oscillator SCN, and found the respiratory exchange ratio, daily activity and circadian period were altered in the *Cdk9*-knockdown mice. Together, our finding designated CDK9 as a novel modulator in circadian clock. CDK9 may serve as a vital basis to understand circadian- and cell cycle-misregulated ailments such as cancer.

1. Introduction

Circadian rhythms coordinate daily physiology and behaviors of living organisms according to the diurnal light-dark cycle [1]. Circadian oscillations rely on transcriptional-translational feedback loops in a cell autonomous manner [1–3]. CLOCK and BMAL1 heterodimerize and act as principal transcription factor to transcriptionally activate clock-controlled genes (CCGs) in a time-dependent manner, by recognizing E-box motif in CCG promotors [4,5]. There are two major interconnected feedback loops participated in the circadian transcriptional regulation. In the primary feedback loop, CLOCK:BMAL1 initiates the transcriptions of *Period* (*Per*) and *Cryptochrome* (*Cry*). When PERIOD and CRYPTOCHROME proteins accumulate to critical levels, they assemble into hetero-complexes and function as corepressors by binding to CLOCK:-BMAL1 further repress their own expression [6]. The repression is later relieved by the degradation of CRYs and PERs over time, renders another cycle of CRYs and PERs expressions taking place [7,8]. In the secondary loop, the nuclear orphan receptors REV-ERBα/β, RORα/β/γ are involved in regulating the temporal expression of BMAL1 and CLOCK. REV-ERBs and RORs are also CCGs under CLOCK:BMAL1 regulation. By recognizing RORE elements in the *Bmal1* and *Clock* promoters, ROR collaborates with PGC-1α to transcriptionally activate *Bmal1* and *Clock* [6,9]. REV-ERB competes for the RORE binding when achieves concentration advantage over ROR with time, and executes as a transcriptional repressor for *Bmal1*, and to lesser extend *Clock* [10,11]. REV-ERB-dependent repression is key to manage circadian oscillations temporally [10,11], thus REV-ERBs are considered as significant targets to manipulate circadian clock at the same time treat circadian-related diseases [12–14]. Interestingly, a cell cycle machinery protein, CDK1, was demonstrated in regulating REV-ERBα stability [15]. This further indicates that circadian clock and cell cycle are coupling processes to drive biological oscillations [16]. Deciphering more of the interconnected mechanisms/targets could help the design of therapeutics on diseases related to deteriorated circadian clock and cell cycle.

Progression of the cell cycle is driven and tightly controlled by several cyclin-dependent kinases (CDKs) forming complexes with their cyclin partners [17]. CDKs serve as key components for cell

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cycle control, DNA repair, and transcriptional regulation [18]. During cell cycle, to warrant the progression with ensured genetic integrity, cells undergo four distinct phases: G0/G1 phase, S phase, G2 phase, and M phase. With respond to mitogenic signals, cells activate cascades of intracellular signaling networks including the expression of cyclin D, to drive transitions from G0 or G1 phase into S phase with essential partners CDK4 and CDK6. Cyclins E accumulates and forms complex with CDK2 during the late G1 phase for the initiation of G1-S transition and DNA replication. Later CDK2 and cyclin A2 help to support the S-G2 transition. Similarly, CDK1 plays a role in the S-G2 transition, subsequently by forming a complex with cyclin B, triggers the G2 phase into mitosis. CDKs also participates in the regulation of RNA polymerase II-based transcription by changing the phosphorylation status at the RNA polymerase II carboxyl-terminal domain (CTD). Significant CDK/cyclin complexes are CDK8/cyclin C, CDK7/cyclin H, CDK9/cyclin T and CDK11/cyclin L for the step-wise activation from pre-initiation, initiation, elongation to RNA processing, respectively [17].

Due to the versatile cellular activities uncovered from the large CDK family members, CDKs are with vast potential to serve as therapeutic targets for malignant diseases such as cancer [19]. Here we study the possible role of new CDK members in circadian regulation, by first examining 17 CDK inhibitors in *mPer2*^{Luc} mouse embryonic fibroblast (MEF) cell line. We found LY2857785 significantly inhibited mPER2:LUC expression, and subsequent siRNA analysis confirmed CDK9 was responsible for the effect. The role of CDK9 in attenuating REV-ERBα may render a new design for modulating circadian period and amplitude.

2. Materials and methods

2.1. Mice

Reporter mouse strain *mPer2*^{Luc} was obtained from the Jackson Laboratory (006852) [20]. All mice were maintained in a standard pathogen-free facility under 12:12 h light-dark cycle. All animal experiments were conducted in accordance with protocols approved by the Institutional Biomedical Research Ethics Committee of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

2.2. Cell culture

HEK293T cell were purchased from ATCC. MEF cells were isolated from E13.5d embryos of wild-type or *mPer2*^{Luc} mice. Cells were cultured in DMEM (Invitrogen) with 10% FBS and 100 U/ml penicillin/streptomycin and maintained at 37 °C with 5% CO_2 in a humidified environment. All cell lines were examined to ensure mycoplasma-free with PCR method (Sigma, MP0035).

2.3. Reagents

CDK inhibitors were obtained from the companies: BS-181, Flavopiridol, LDC000067 and LY2857785 were purchased from Med Chem Express. BMS-265246, JNJ-7706621, Milciclib, Palbociclib and PHA-767491 were purchased from Selleck. Bohemine, Kenpaullone, LY2835219, MK-8776, PHA-793887, Ribociclib, RO-3306 and Roscovitine were purchased from Targetmol. Antibodies were purchased from the following resource: anti-BMAL1 (Cell Signaling Technology, 14020), anti-CDK9 (Santa Cruz, 13130), anti-CLOCK1 (Cell Signaling Technology, 5157), anti-CRY1 (Abcam, 54649), anti-GAPDH (ProteinTech, 60004), anti-PER2 (ABclonal, 13168), anti-REV-ERBα (Cell Signaling Technology, 13418), and HRP-goat anti-rabbit secondary antibody (Bio-Rad, 1706515).

2.4. Small molecule treatment

The primary chemical tests were carried out in a 384 well format, with approximately 3000 *mPer2*^{Luc} MEF cells seeded 24 h prior to the chemical applications at 10 μM dosage. After 24 h of treatment, luciferase activities were determined with ONE-Glo™ luciferase assay (Promega). After luminescence measurement, cell viability was determined by alamarBlue assay (Invitrogen). The summary of relative compound effect was listed in Fig. 1A.

2.5. Real-time bioluminescence recording

For CDK inhibitor test, approximately 2.5×10^5 of *mPer2*^{Luc} MEF cells were seeded and cultured for 48 h in 3.5 cm dishes until confluent. The cells were then synchronized with 200 nM dexamethasone for 1 h, before the bioluminescence recording in serum free DMEM medium supplement with 200 μM luciferin together with CDK inhibitors. The dishes were transferred and recorded in a light-tight chamber containing a PMT detector assemblies (Actimetrics) for continuous 4 days. Results were processed with the ClockLab software for period and amplitude.

2.6. siRNA knockdown

Silencer oligonucleotides for *Cdk2*, *Cdk5*, *Cdk7*, *Cdk8*, *Cdk9* and scramble controls were purchased from GenePharma (Table S1). *mPer2*^{Luc} MEF cells were transiently transfected with *Cdk* siRNA oligonucleotides using Lipofectamine 3000 (Invitrogen), and were subjected to real-time bioluminescence recording as described above. The knockdown efficiencies were determined at 24 h post transfection by real-time RT PCR. Primers for *Cdks* and a ribosomal reference gene, *Rpl19* were listed in Table S1.

2.7. Immunoprecipitation

293T cells were transfected with plasmids for the expressions of CDK9 or REV-ERBα for 48 h 1×10^7 293T cells were then harvested and lysed with 400 μl chilled lysis buffer supplemented with protease inhibitors as mentioned. Approximately 3 mg cell lysates were then incubated with 20 μl anti-Flag M2 magnetic beads (Sigma) for 4 h at 4 °C. The pellet was washed with TBS buffer for three times, before the elution of protein complex from the beads by 100 μl of 3X FLAG peptide (150 ng/μl, Sigma). The eluted samples were subjected for immunoblot analysis.

2.8. Electric mobility shift assay

EMSA was performed using a chemiluminescent EMSA Kit (Beyotime), and the putative RORE probe of *Bmal1* were annealed and labeled with biotin according to manufacturer's recommended procedures. The sequences of the probes utilized in the EMSA are:

*mBmal1*_RORE_F: GAAGGCAGAAAGTAGGTCAGGGACGGAG
*mBmal1*_RORE_R: CTCCGTCCCTGACCTACTTTCTGCCTTC

For RORE binding assay, nuclear extracts from HEK293T cells were prepared as described above, and incubated with RORE probe for 40 min at room temperature prior to electrophoresis with 4% native PAGE. 0.3 μg anti-REV-ERBα antibody were mixed with 5 μg lysate to deplete REV-ERBα to examine the binding RORE efficacy.

2.9. Stereotaxic virus injection and animal studies

8 week-old male C57BL/6 mice were anaesthetized then placed into a stereotaxic apparatus (51615, Stoelting). Bilateral injection of

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300 nl AAVs (rAAV-DJ/H1-sh*Cdk9*-CAG-EGFP or vector control, Taitool Bioscience) at suprachiasmatic nucleus (SCN). Coordinates Bregma A/P: −0.46 mm; D/V: −5.5 mm, M/L: ±0.2 mm were applied. Mice were allowed to recover for two weeks, then transferred to metabolic cage (CLAMS-16, Columbus) to measure the activity and respiratory exchange ratio for three days.

2.10. Immunohistochemistry

Mice were euthanized then perfused with 4% paraformaldehyde, subsequently immersed in 30% sucrose before embedded in OCT. 30 μm brain sections were collected and treated with 3% H_2O_2 to quench the residual peroxidase activity. Antigen retrieval were achieved by 0.05% trypsin digestion for 5 min at room temperature. Brain sections were subsequently blocked with TNB blocking buffer prior to primary antibodies (anti-CDK9: 1:50; anti-REV-ERBα: 1:500 diluted), and HRP anti-rabbit secondary antibody (1:1000 diluted) incubation. Staining signal was amplified with TSA kit (PerkinElmer), then visualized by fluorescence microscopy (Axio Imager A2, ZEISS). Software ImageJ was used to analyze and quantify the microscopy results.

2.11. Statistics

In vitro experiments were performed at least three times unless otherwise indicated. Statistical analyses were performed using Prism version 7.0 (GraphPad) with an unpaired two-tailed Student's *t*-test. Statistical significances are labeled as ns (not significant); $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. CDK inhibitors BS-181 and LY2857785 damp mPER2::LUC expression

To investigate the potential roles of CDKs on circadian control, we applied the mPER2:LUC fusion as a reporter to track CDKs effects on PER2 oscillation [20]. We tested *mPer2*^{Luc} MEF cells with 17 commonly used CDK inhibitors for cell viability assay, to ensure the condition did not cause extensive cell loss post 24-h treatment. At the same time, we measured the respective luciferase activities to determine the small molecule effect on mPER2:LUC expression (Fig. 1A). We found that among the inhibitors, BS-181 and LY2857785 were most effective in suppressing mPER2:LUC amplitude (Fig. 1A). BS-181 is a selective CDK7 inhibitor that blocks the activity of CDK7 with an IC_{50} of 21 nM. It also inhibits CDK2 and CDK5 at higher concentrations, 880 nM and 3 μM, respectively [21]. In our assay, BS-181 inhibited mPER2:LUC activity at the concentration of 10 μM (Fig. 1B), suggesting that the suppression may derive from the synergistic effect of CDK2, 5 and 7. Extended treatment of BS-181 caused lengthened PER2 period as revealed by the real-time luminescence recording (Fig. 1C). LY2857785 was particularly effective in our test. LY2857785 led to a mild inhibition of mPER2:LUC oscillation at low concentration 40 nM, and complete inhibition in a broad operative range from 0.37 to 10 μM (Fig. 1B). LY2857785 is a potent inhibitor designed for CDK9 (IC_{50} = 11 nM), at the same time inhibits CDK7 and CDK8 with IC_{50} of 246 nM and 16 nM, respectively [22,23]. Extended treatment of LY2857785 blunted PER2 oscillation in the real-time luminescence assay (Fig. 1C).

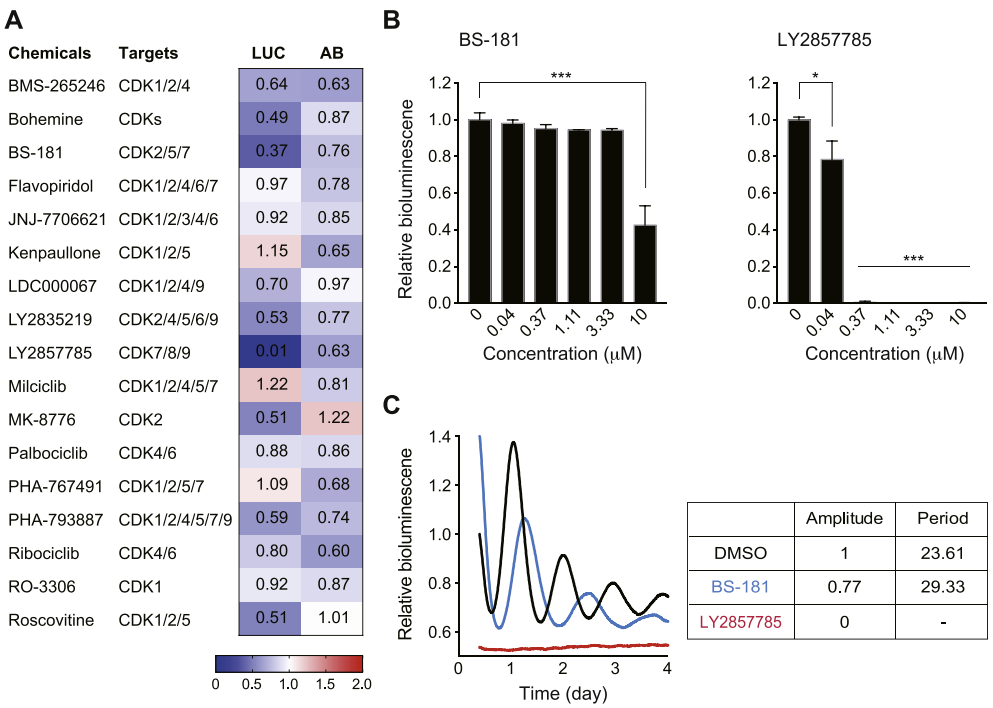

Fig. 1. CDK inhibitors BS-181 and LY2857785 modulate circadian amplitude. (A) Heat map summary of the relative mPER2:LUC bioluminescence results from MEFs treated with 17 commonly used CDK inhibitors. LUC indicates the relative mPER2:LUC bioluminescence activity level, and AB indicates the relative viability to DMSO control determined by alamarBlue assay. (B) Dose-response test of BS-181 and LY2857785 in the *mPer2*^{Luc} MEFs. Bioluminescence activities were measured post 24 h treatment. Data are shown as mean ± s.d. (C) Real-time bioluminescence recordings of synchronized *mPer2*^{Luc} MEF cells with DMSO (black curve), BS-181 (10 μM, blue curve), or LY2857785 (10 μM, red curve) treatment. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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3.2. CDK9 knockdown mimics LY2857785 effect on Per2 down-regulation

To verify the responsible CDKs in regulating circadian clock, we next performed knockdown analysis to validate *Cdk2*, *Cdk5*, *Cdk7*, *Cdk8* and *Cdk9* independently, with siRNAs designed specifically in these genes (Table S1). The knockdown efficiencies were in the range of 50–77%, examined by qPCR analysis (Fig. 2A). The siRNA transfected *mPer2*^{Luc} MEFs were further subjected to real-time luminescence recording for continuous 3 days. Among the candidates, only *Cdk9* knockdown resulted in clear mPER2:LUC amplitude change, for ~39% reduction in the luminescence activity (Fig. 2B and C). Other siRNA-targeted *Cdks* were inseparable from the scramble control for both amplitude and period outcomes (Fig. 2C). Cells upon LY2857785 treatment expressed higher level of *Rev-erbα*, at the same time with lowered levels of *Bmal1*, *Clock* and *Per2* (Fig. 2D). Similarly, the *Cdk9* knockdown also brought about 2-fold increase of *Rev-erbα* ($P < 0.001$), and significant decrease of *Bmal1*, *Cry2* and *Per2* transcripts (Fig. 2E). Immunoblots of MEF samples also revealed a clear elevation of REV-ERBα upon *Cdk9* knockdown (Fig. 2F), in accordance with the result that the acrophase of REV-ERBα at zeitbeber time ZT6-ZT10 was correlated to CDK9 trough in the liver samples (Fig. 2G). Together, the results implicated the loss of CDK9 may trigger a dysregulated *Rev-erbα* level, in turn suppressed core clock genes including *Bmal1* and *Per2*.

3.3. CDK9 interacts with REV-ERBα and regulates the binding to RORE

Immunoprecipitation experiments mediated by FLAG M2 pull-down demonstrated that CDK9 interacted with REV-ERBα when co-expressed in the HEK293T cells (Fig. 3A). A reciprocal IP with overexpressing REV-ERBα-FLAG also pulled CDK9 as an associate protein, both in the conditions of overexpressed V5-tagged CDK9 and endogenous CDK9 (Fig. 3B and C). REV-ERBα represses *Bmal1* transcription through binding to the ROR/REV-ERB-response element (RORE) [11]. To assay the role of CDK9 in modulating the REV-ERBα-mediated transcriptional regulation, we applied an electrophoretic mobility shift assay (EMSA) designed at the *Bmal1* RORE sequence for REV-ERBα binding to evaluate the binding efficiencies upon CDK9 depletion. We found *Cdk9* knockdown facilitated RORE occupancy (Fig. 3D). The effect appeared to be REV-ERBα dependent, as the depletion of REV-ERBα largely lowered the binding efficiency (Fig. 3E). In summary, CDK9 acts as a suppressor for the binding of REV-ERBα to RORE.

3.4. Knockdown Cdk9 at the SCN leads to circadian period change

To investigation the role of CDK9 in circadian regulation *in vivo*, we knocked down *Cdk9* at the central circadian oscillator SCN by using adeno-associated virus (AAV)-mediated expression of shRNA

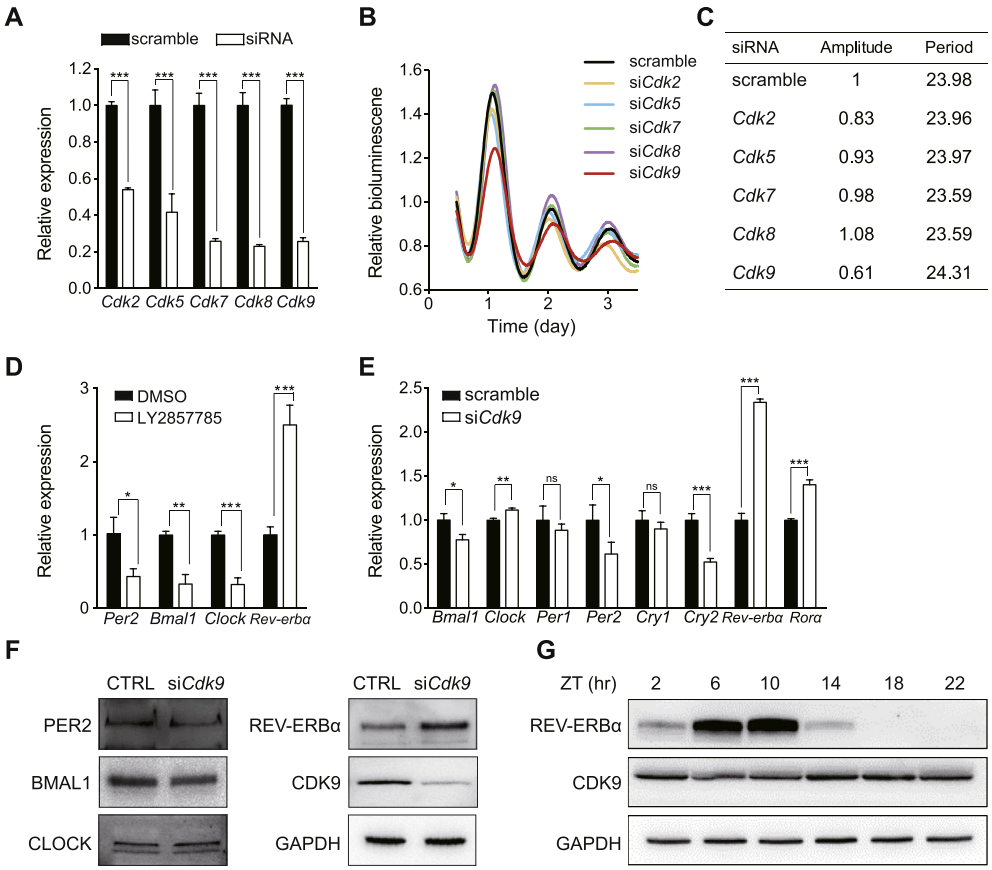

Fig. 2. CDK9 knockdown resembles LY2857785 effect on circadian modulation. (A) Real-Time PCR analysis for the knock-down efficiencies of *Cdk2*, *Cdk5*, *Cdk7*, *Cdk8* or *Cdk9* siRNA-treated *mPer2*^{Luc} MEF cells. Scramble siRNA was applied as the control. (B) Real-time bioluminescence recordings of synchronized *mPer2*^{Luc} MEF cells upon siRNA treatment of *Cdk2*, *Cdk5*, *Cdk7*, *Cdk8* or *Cdk9*. Scramble siRNA was applied as the control and indicated in black. (C) Amplitude and period analyses as summarized from (B). (D)Real-Time PCR analysis of core clock gene mRNA levels after 24 h LY2857785 treatment. (E) Real-Time PCR analysis of core clock gene mRNA levels after 24 h si*Cdk9* treatment. (F) Immunoblots of core clock protein levels from scramble and si*Cdk9* treated MEF cells. (G) Immunoblots of REV-ERBα and CDK9 from wild-type mouse liver lysates collected at different zeitgeber time points. GAPDH was used as the loading control. Data are shown as mean ± s.d.

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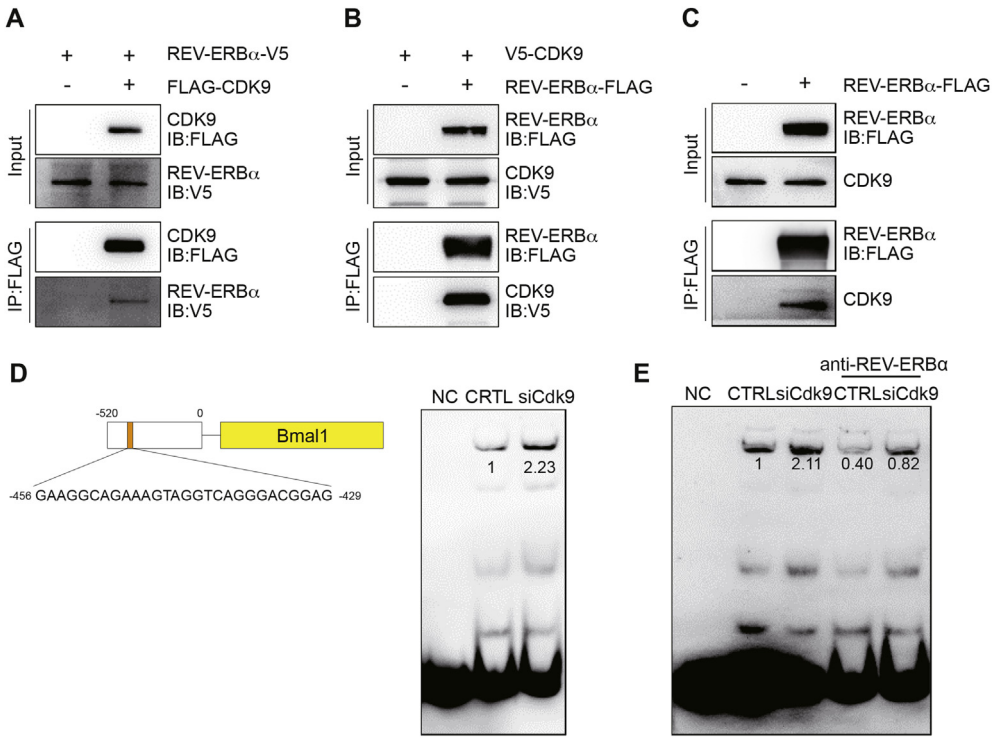

Fig. 3. CDK9 associates with REV-ERBα to attenuate the binding to RORE. (A) FLAG-mediated co-immunoprecipitation of FLAG-CDK9 and REV-ERBα-V5 from HEK293T cell lysates post 48 h transient expression. (B) FLAG-mediated co-immunoprecipitation of REV-ERBα-FLAG and V5-CDK9 from HEK293T cell lysates post 48 h transient expression. (C) Co-immunoprecipitation of endogenous CDK9 by REV-ERBα-FLAG. (D) A schematic representation of the *RORE* site in the *Bmal1* promoter that is recognized by REV-ERBα (left). Electrophoretic mobility shift assay of biotin-labeled *RORE* samples without nuclear lysate control (NC), nuclear lysate of treating scramble siRNA (CRTL), or nuclear lysate of treating si*Cdk9* (right). REV-ERBα binding efficiencies were quantified and labeled. (E) Depletion test via anti- REV-ERBα antibody, followed by electrophoretic mobility shift assay as in (D).

targeting *Cdk9*. We noticed an increase of respiratory exchange ratio (RER) in the *Cdk9*-deficient mice, especially at the early day during resting, indicated an extended energy utilization toward carbohydrate (Fig. 4A). Analysis of RER peaks and troughs revealed that the diurnal RER periods were extended in *Cdk9*-deficient mice (Fig. 4B). Further, the locomotor activities of *Cdk9*-deficient mice were significantly reduced, chiefly during the dark time (Fig. 4C). The down-regulated CDK9 expression in the SCN also resulted in an elevated REV-ERBα level (Fig. 4D), pointed out that CDK9 is a key modulator for regulating REV-ERBα level *in vitro*, in the liver (Fig. 2F and G), and in the SCN (Fig. 4D). Together, our data suggested the prospective of targeting CDK9 for the manipulation of circadian clock period and amplitude.

4. Discussion

In the current study, we found CDK9 as a new CDK member involved in circadian control, and identified a compound LY2857785 that can enhance REV-ERBα expression through CDK9 inhibition. LY2857785 exerted noticeable inhibitory activity on PER2 expression at the concentration of 40 nM, and blunted PER2 oscillation at 370 nM. Knock-down *Cdk9*, but not *Cdk7* or *Cdk8*, resembled LY2857785 effect on repressing PER2 oscillation, as well as circadian clock gene expression including *Bmal1* and *Cry2*. Of note, CDK9 suppressed REV-ERBα levels and mitigated REV-ERBα binding to the *Bmal1* RORE, implicated the temporal role of CDK9 in circadian modulation. Further, mice that were knocked-down *Cdk9* in the SCN showed reduced locomotor activities and extended RER period. The higher RER level at early day (resting) time indicated the prolonged energy utilization toward carbohydrate, in addition supported the role of CDK9 in circadian temporal regulation.

REV-ERBs are key regulators that coordinate metabolic programs and circadian rhythm [10,15,24]. Small molecules have been identified to directly target REV-ERBs for pharmacologically manipulating circadian rhythm and metabolic programs [12], as well as the application in anticancer therapeutics [14]. In our study, we found that LY2857785 acts as an indirect agent to facilitate REV-ERBα function through the liberation of REV-ERBα from CDK9. The consequence is similar to a recently reported compound KK-S6, which appeared to enhance the repressive activity of REV-ERBα directly on *Bmal1* RORE [13]. Our finding added a novel candidate, CDK9, as a potential target to tune RORE-mediated transcriptional activity. The phenotypes observed in mice with *Cdk9* knock-down in the SCN offered a likely design of CDK9-related therapeutics for circadian disorders both in the central and in peripherals.

Another Cyclin-dependent kinase, CDK1 has been demonstrated to mark REV-ERBα for FBXW7-mediated degradation [15], thus contributes in line with the effect of CDK9 in antagonizing REV-ERBα. That is interesting that the evolutionally conserved kinases, originally found to regulate cell-cycle progression, participate actively in circadian control. Different from CDK1, which forms complexes with cyclin A or cyclin B to trigger S—G2 transition and G2 progression to M-phase, respectively [25]; CDK9 regulates RNA polymerase II-based transcription at the elongation step [26]. Whether the canonical cell-cycle regulator, such as CDK1, and the auxiliary transcription elongation factor CDK9 work in proximity during circadian modulation require further investigation. RNA polymerase II activity has been recognized to tightly accompany with the circadian cycles [27,28]. RNA polymerase II recruitment, and the associated chromatin states assist to build a time-dependent pattern coordinate with the circadian clock [29,30]. An intriguing point that CDK7 and CDK8, two other potential targets of

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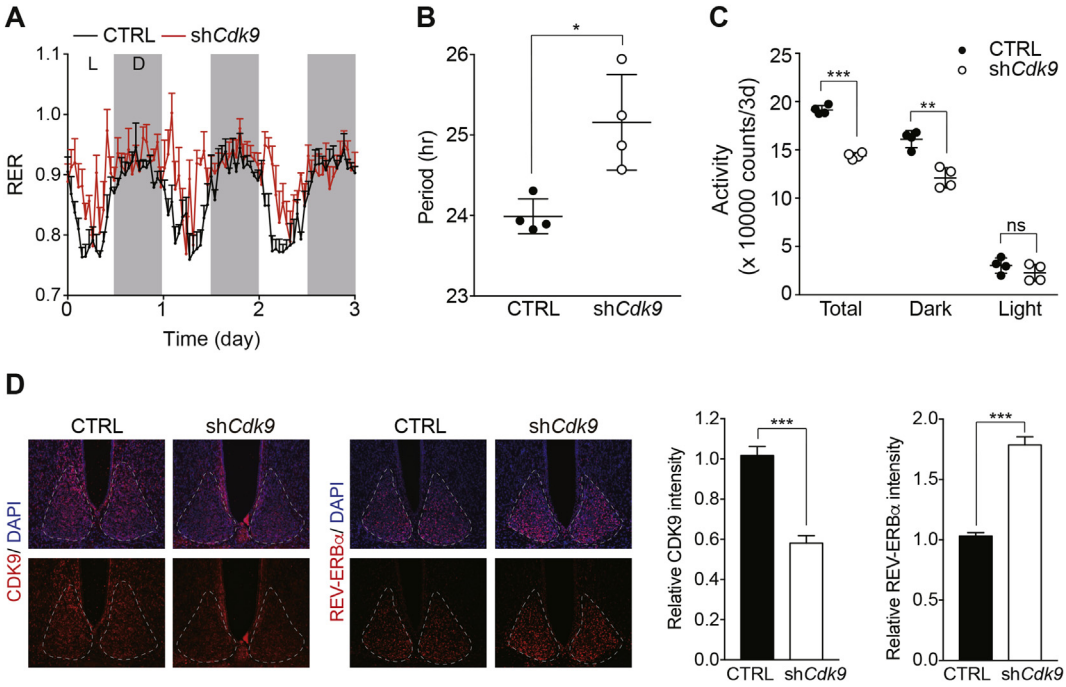

Fig. 4. AAV-mediated knockdown of *Cdk9* at the SCN leads to circadian period and RER change. (A) Respiratory exchange ratio (RER) of control (black) and AAV-sh*Cdk9* (red) mice (n = 4). Note that the knock-down animals showed higher RER in the early light phase. (B) Circadian period, and (C) activity levels analyzed from the metabolic cage data. Results were processed with the ClockLab software. (D) Immunohistochemistry of CDK9 (left) and REV-ERBα (right) in SCN. Data are shown as mean ± s.e.m. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

LY2857785, and are as well involved in RNA polymerase II-based transcriptional regulation, did not influence mPER2:LUC oscillation. CDK7 and CDK8 participate in the RNA polymerase II-initiation step [17]. Whether it is the elongation process, as represented by CDK9, serves as a key step of RNA polymerase II for modulating circadian cycles, or there are other factors involved in the initiation step to mark the rhythmic processes remained to be investigated.

Taken together, the consistent regulatory activity of CDK9 on REV-ERBα *in vitro*, in the liver and in the SCN implicated CDK9 to be a promising candidate in modulating circadian period and amplitude. Here we also proposed a preclinical anticancer CDK9 inhibitor LY2857785 as a potent circadian regulating agent. LY2857785 and its related compounds could be useful for new therapeutic designs in circadian disorders and malignant diseases such as cancer.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.04.043.

Appendix A. Supplementary data

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