CIRCADIAN RHYTHM

Chemical perturbations reveal that RUVBL2 regulates the circadian phase in mammals

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Transcriptional regulation lies at the core of the circadian clockwork, but how the clock-related transcription machinery controls the circadian phase is not understood. Here, we show both in human cells and in mice that RuvB-like ATPase 2 (RUVBL2) interacts with other known clock proteins on chromatin to regulate the circadian phase. Pharmacological perturbation of RUVBL2 with the adenosine analog compound cordycepin resulted in a rapid-onset 12-hour clock phase-shift phenotype at human cell, mouse tissue, and whole-animal live imaging levels. Using simple peripheral injection treatment, we found that cordycepin penetrated the blood-brain barrier and caused rapid entrainment of the circadian phase, facilitating reduced duration of recovery in a mouse jet-lag model. We solved a crystal structure for human RUVBL2 in complex with a physiological metabolite of cordycepin, and biochemical assays showed that cordycepin treatment caused disassembly of an interaction between RUVBL2 and the core clock component BMAL1. Moreover, we showed with spike-in ChIP-seq analysis and binding assays that cordycepin treatment caused disassembly of the circadian super-complex, which normally resides at E-box chromatin loci such as *PER1*, *PER2*, *DBP,* and *NR1D1*. Mathematical modeling supported that the observed type 0 phase shifts resulted from derepression of E-box clock gene transcription.

INTRODUCTION

The circadian clock is tightly linked to human health. Clock malfunctions result in sleep disorders and lead to other diseases (*1*–*4*), and a recent study emphasized that circadian timing must be considered when clinicians select the dosage regime for a given medicine (*5*). The eukaryotic clock consists of a core oscillator that regulates its own transcriptional activity via negative feedback, generating a roughly 24-hour rhythm in cells, tissues, and whole organisms (*6*). There are multiple associated transcriptional feedback loops that engage with the core oscillator to form a nested network that hierarchically regulates physiological rhythms throughout the organism (*7*, *8*).

In mammals, well-characterized core clock components include the transcription factors BMAL1/CLOCK and their specific repressor Periods 1–3/Cryptochromes 1–2 (PER/CRY). According to the "transcriptional/translational feedback loop" (TTFL) model, BMAL1 and CLOCK form a heterodimer and bind to E/E′-box sequences in gene promoters, initiating transcription of genes including *PER/CRY*. When the translated PER/CRY proteins accumulate, they inhibit BMAL1/CLOCK transcriptional activity. These interactions together generate a delayed feedback loop that oscillates with an approximately 24-hour cycle (*1*, *9*). The TTFL model provides a dynamic view of how clock components work together to generate circadian oscillation.

Three parameters are used to describe the general process of oscillation: period, amplitude, and phase (*6*). Most of the previous investigations of circadian oscillation have focused on the clock period, because it is generally thought to be the most robust (that is, resilient to perturbation by environmental factors) of the three parameters and is thought to be directly related to the core regulation of the clock (*10*). Only a few studies have focused on the amplitude of circadian oscillations, but it is notable that at least two studies have implicated the activity of the Clock/dClk protein in the regulation of amplitude (*11*, *12*). Using high-throughput screens, even fewer studies have examined the phase of the clock, which is very sensitive to environmental perturbations (*10*, *13*). Nevertheless, it has been recognized that the phase may actually be equally, if not more, important for the clock than either the period or amplitude, as the phase of entrainment defines the regulatory control points for circadian transcription and determines the time at which the clock oscillation begins (*14*). Here, we used a new chemical screening strategy with stringent criteria to monitor clock phase phenotypes to identify a group of small molecules that robustly and predictably shift the clock phase.

RESULTS

Identification of cordycepin as a circadian phase shifter

To identify small molecules that regulate the mammalian clock phase, we conducted a chemical screen using human osteosarcoma U2OS cells harboring a luciferase gene under the control of either the *Per2* or *Bmal1* promoter (*Per2-dLuc* or *Bmal1-dLuc* cells). Such cells were successfully used in a previous small interfering RNA

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(siRNA) screen for clock modifier genes (*15*). We optimized our screening strategy by simultaneous use of both *Per2-dLuc* and *Bmal1-dLuc* U2OS reporter lines; note that previous chemical screens for clock modifier compounds used only *Bmal1-dLuc* cells (*16*–*18*). Furthermore, we set the threshold for phase change phenotypes to greater than 4 hours and set the requirement that a hit compound must not cause any reduction in clock amplitude in either of the cell lines.

We screened an institutional pilot chemical library comprising ~10,000 National Institutes of Health (NIH) clinical/preclinical trial drugs (*19*), purified natural product compounds, and synthetic compounds. Previous studies established that the synthetic glucocorticoid analog dexamethasone (dex) modifies the clock phase (*20*). Consistent with this, we found that a group of dex-like drugs delayed the phase of U2OS cells by 4 hours (fig. S1A). We also found a nucleoside compound—3′-deoxyadenosine (cordycepin)—that caused a robust and reliable "antiphasic" phenotype, completely reversing (a 12-hour phase shift) the expression pattern of clock genes (Fig. 1A). This phase-shift phenotype appeared to be unrelated to other known signals that affect the clock phase [cyclic-adenosine 3′,5′-monophosphate (cAMP), Ca^{2+} pathways] or transcription in general (fig. S1, B to D).

To evaluate the possibility that compounds similar to cordycepin may also shift the clock phase, we tested 117 structurally related nucleosides using the same experimental system (Fig. 1, B and C) and found three additional nucleosides that caused a phase-shift phenotype. All three compounds were adenosine derivatives: N6-benzyl-adenosine, 3-deaza-adenosine (DAA), and 7-deaza-adenosine (tubercidin) (Fig. 1C). Nucleosides sharing the sugar moieties with these hits but having different nucleobases (for example, uridine) did not shift the clock phase [Fig. 1B and fig. S2 (*21*, *22*)].

Despite this apparent specificity of adenosine derivatives in the observed phase shift, such an activity did not seem to be mediated by the adenosine receptor signaling pathway, as naïve adenosine did not shift the clock phase at all (Fig. 1C). Consistent with this, treating the cells with a cocktail of antagonists of pan-adenosine receptors did not cause a phase shift, nor could cotreatment rescue the phase-shift phenotype caused by cordycepin (fig. S3, A and B). Rather, blocking the transport of adenosine into cells greatly attenuated the phase-shift phenotype of cordycepin; thus, we conclude that the phase shift resulted from the intracellular activity of the adenosine derivatives per se (fig. S3C). Moreover, saturating the adenosine receptors by overdosing the experimental system with an excess of naïve adenosine prevented any shifting of the clock phase upon cordycepin treatment (Fig. 1, D and E).

Our experiments also showed that cordycepin has a short half-life, as the cordycepin concentration in the cells was very low 4 hours after administration (Fig. 1E). The phase-shifting effect of cordycepin was dependent on the time of administration: The shape of the phase response curve following cordycepin administration showed a strong phase-shift (>6 hours) phenotype, which is categorized as the "type 0–resetting," where there is a discontinuity existing at the transition between phase advance and delay (Fig. 1F and data file S1) (*23*). Further, and supporting an influence for the cellular metabolism of cordycepin, when we pretreated cells with pentostatin—an adenosine-deaminase inhibitor used commonly in studies of adenosine signaling (*24*)—we detected a large (128×) increase in the phase-shift potency of cordycepin (Fig. 1C and data file S2). Although treatment with pentostatin alone had no effect on the clock, we again observed very different potencies for combined treatments comprising pentostatin and various adenosine analogs (fig. S3D), which pointed to an apparently major phase-shifting influence for the amino group of the purine ring.

Cordycepin-induced clock phase shifts in tissues and animals

Having demonstrated the ability of cordycepin to specifically induce a phase shift in cells, we next conducted bioluminescence recording assays with ex vivo cultures of livers and suprachiasmatic nuclei (SCN), the master organ of the clock, from PER2::LUC knock-in mice, and confirmed that cordycepin was able to reverse the PER2 phase in this condition (fig. S4). Because adenosine can pass through the blood-brain barrier (BBB) via ABC/nucleotide transporters located in glial cells (*25*), we administered the adenosine analog cordycepin to rats by intraperitoneal injection. We subsequently detected cordycepin in rat cerebrospinal fluid (CSF) at concentrations consistent with previous pharmacokinetic studies (*26*) and additionally observed a rapid decline in the cordycepin concentration over the course of 30 min (fig. S5).

The fact that cordycepin can pass the BBB prompted us to conduct locomotor assays to evaluate whether this phase-shift compound can induce behavioral effects in animals. In the gold standard mouse behavioral assay for jet-lag, wild-type (WT) C57BL/6J mice typically take ~10 days to adapt to an 8-hour phase advance (*27*). This adaption time to a phase advance was significantly shortened ($P < 0.001$) to only 4 days when a cordycepin injection was administered at Zeitgeber time 11 (ZT11; light off 1 hour later at ZT12) on each of the first 2 days (Fig. 2A). Similarly, the cordycepin-treated mice adapted significantly faster ($P < 0.001$) to an 8-hour phase-delay assay (Fig. 2B). Consistent with our results in cells highlighting the importance of cordycepin administration time, we also found that the mice did not show an accelerate behavioral phase-shift phenotype when cordycepin was injected at ZT23 (1 hour before light on; fig. S6). These results establish that the intraperitoneal administration of cordycepin at an appropriate time can shift the phase of the mammalian brain clock and can alter locomotor behavior.

Identification of RUVBL2 as the cordycepin target that shifts the clock phase

To identify the direct target of cordycepin, we applied a chemical-genetic approach combined with immunoprecipitation-coupled mass spectrometry (IP-MS) (fig. S7). Given that the clock phenotype is likely involved in the regulation of the transcription of clock genes, we used siRNA libraries containing 1530 transcription factors and 463 epigenetic factors to evaluate which gene(s) may be functionally related to cordycepin-mediated *Per2-dLuc* perturbation. Knockdown of each of these 18 genes abolished the cordycepin-mediated induction of *Per2-dLuc* (fig. S7A and data file S3). To identify proteins that physically interact with cordycepin, we conducted an IP-MS assay with U2OS cell lysates using functionally active biotinylated cordycepin as bait (fig. S7B). The IP-MS assay identified 81 potential cordycepin-binding partners (data file S4). By combining the 18 hits from the knockdown experiment with the 81 hits from these physical interaction assays, we found that there was only one common hit: RuvB-like adenosine triphosphatase (ATPase) 2 (*RUVBL2*). Specifically, *RUVBL2* mediated cordycepin-induced circadian phase shift as confirmed by siRNA knockdown (Fig. 3A

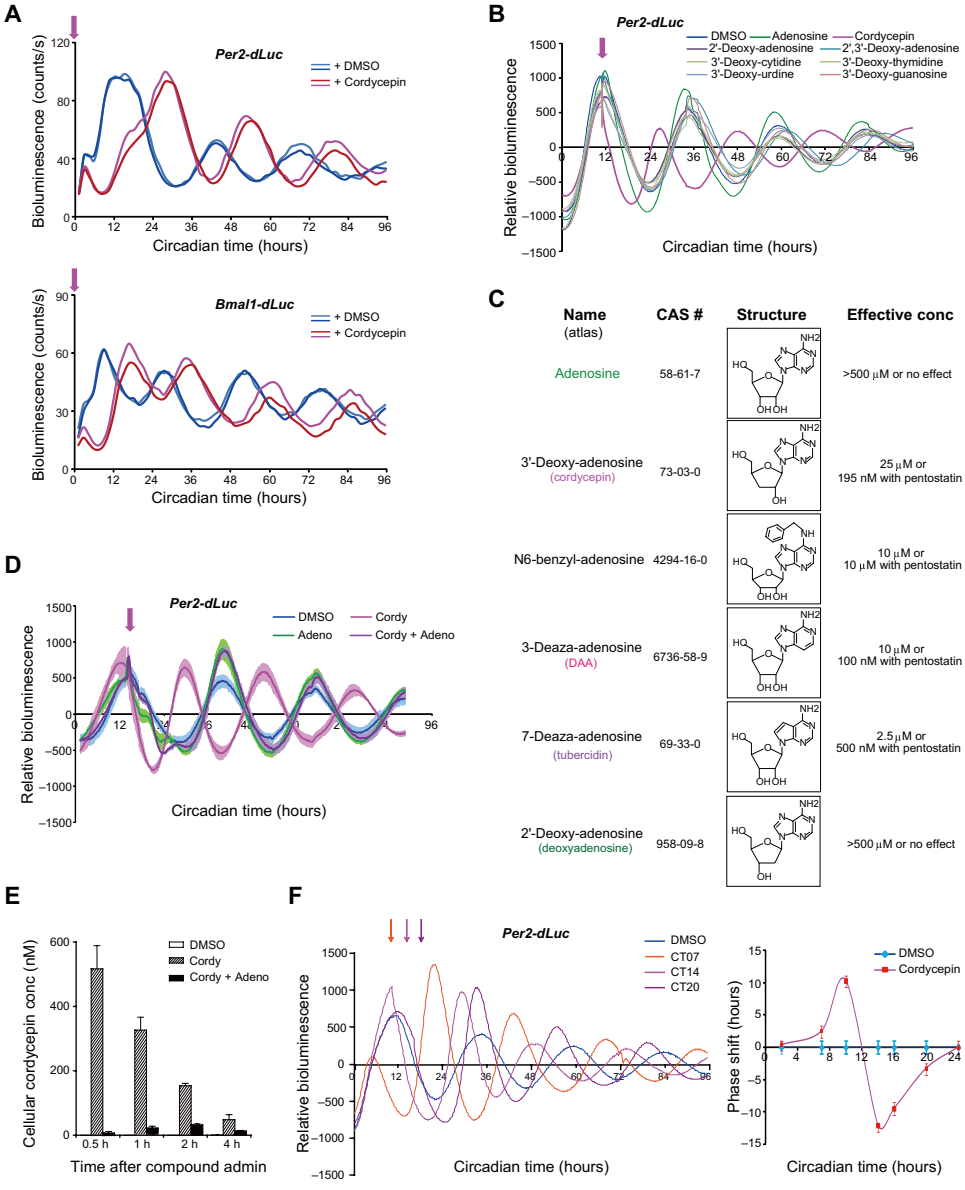

Fig. 1. Discovery of cordycepin as a clock antiphasic compound. (**A**) Bioluminescent recordings of *Per2-dLuc* and *Bmal1-dLuc* U2OS cells upon cordycepin treatment (25 μM). Duplicate results were shown here, and DMSO treatment was used as control. (**B**) Effects of cordycepin (25 μM) and other indicated nucleosides on reporter rhythms in *Per2-dLuc* U2OS cells. For the other nucleosides, both 25 and 500 μM concentrations were tested. For clarity, the data shown here are representative of triplicate experiments (25 μM; $n = 3$); detailed analyses using the BioDare2 program are presented in fig. S2. (**C**) Chemical structures and effective doses of the only four antiphasic hits from a library consisting of 118 nucleosides. These four compounds are all adenosine analogs. (**D**) Effect of excessive adenosine (Adeno, 500 μM) on cordycepin (Cordy, 25 μM) on the circadian phase in *Per2-dLuc* U2OS cells. The dark lines of the traces represent the means, and the adjacent lighter areas indicate the SDs of the triplicate samples ($n = 3$). (**E**) Intracellular concentrations of cordycepin in treated U2OS cells ($n = 3$). Data are mean ± SD. (**F**) Left: Bioluminescent recordings of *Per2-dLuc* U2OS cells upon cordycepin treatment (25 μM) at indicated time. Data shown here are representative of triplicate experiments ($n = 3$). Right: A phase-responsive curve following cordycepin treatment. $n = 4$ for each time point; data are presented as mean ± SD. The full data and BioDare2 analysis results are in data file S1. Arrows (in A, B, D, and F) indicate the time when administration of drugs; CT0 was set at medium change; for the experiments carried out in a Lumicycle (B, D, and F), CT0 was 24 hours post-medium change.

function in transcriptional regulation (*28*, *29*).

To assess whether cordycepin can physically interact with RUVBL2, we conducted competition pull-down assays. In the absence of unaltered cordycepin, biotinylated-cordycepin (which can still function as a phase shifter with less potency; fig. S7C) pulled down RUVBL2 both from U2OS cell lysates and as a purified recombinant protein; however, when outcompeted by various excess concentrations of unaltered cordycepin, the biotinylated compound did not pull down RUVBL2 (Fig. 3B). Furthermore, a surface plasmon resonance (SPR) assay showed that purified RUVBL2 binds to cordycepin with a K_d value of about 500 nM (fig. S7D). Together, these results indicate that RUVBL2 is a direct target of cordycepin.

To examine the expression pattern of RUVBL2 in the brain, where cordycepin may contribute to regulating locomotor behavior, we performed an immunofluorescence staining assay using an anti-RUVBL2 antibody. We observed widespread expression of this protein in the brain, with especially pronounced expression in layer II of the piriform cortex and the SCN of the hypothalamus (Fig. 3C). The expression of RUVBL2 in the SCN also followed a clear circadian pattern, peaking at CT10 (fig. S9). This observation is consistent with the online database CircaDB for circadian studies (*30*), showing that both *Ruvbl1* and *Ruvbl2* are faithfully rhythmic in mouse liver (fig. S10A). Furthermore, when we closely examined data from a landmark chromatin immunoprecipitation sequencing (ChIP-seq) study (*31*), we found that the promoter region of the mouse *Ruvbl2* gene was directly and rhythmically bound by core clock components including BMAL1, CRY1, and CRY2 (fig. S10B), thereby establishing that the expression of *Ruvbl2* is controlled by the circadian clock in vivo.

To validate whether RUVBL2 regulates the clock, we searched our previous genome-wide siRNA study (*15*) and found that knockdown of *RUVBL2* disrupted the clock, illustrating its essentiality for proper clock function in U2OS cells (fig. S11). In addition, knockdown of multiple subunits of the RUVBL-containing TIP60 (*KAT5*) transcription regulatory complex caused a remarkable clock phenotype (fig. S11, A and B), suggesting that the TIP60 complex may be

and fig. S8). We note that RUVBL2 is an AAA-type ATPase (ATPase associated with diverse cellular activities) with DNA helicase activity and is also a core component of the TIP60 complex known to

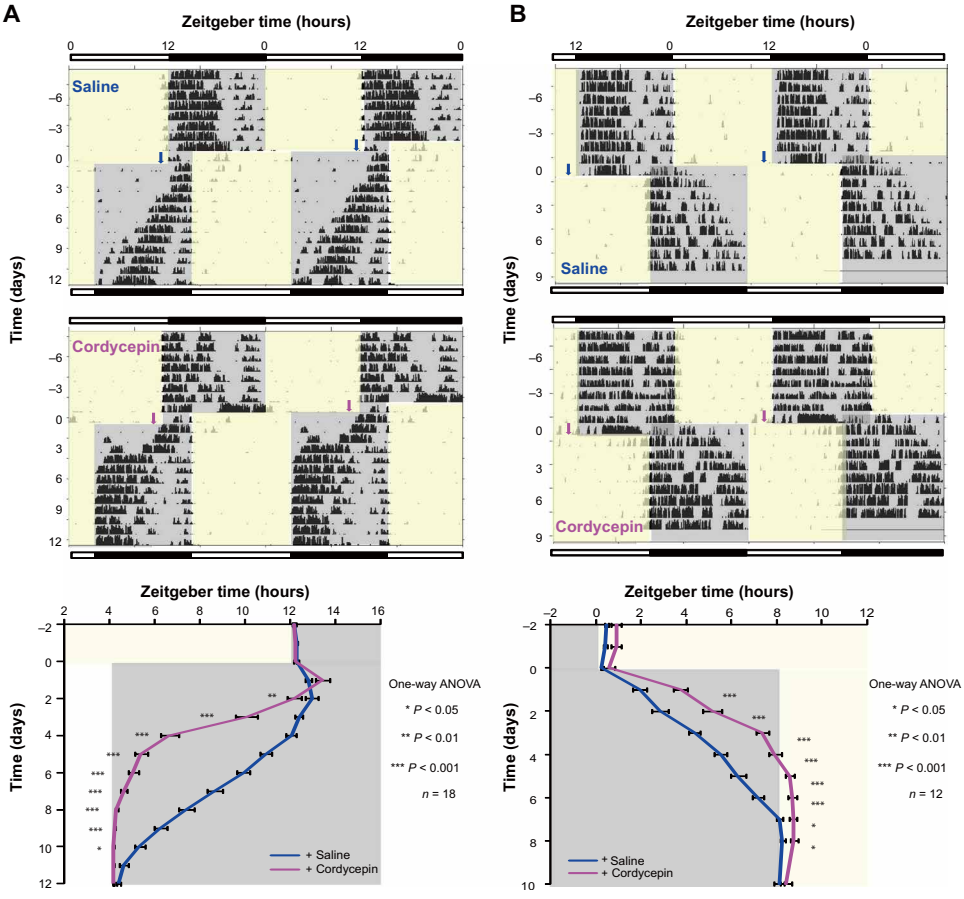

Fig. 2. Regulation of the murine locomotor phase by peripheral administration of cordycepin. Top: Double plots show mouse locomotor assays for the phase advance (**A**) and phase delay (**B**) experiments. Bottom: Statistical analysis of the onset of locomotor activity of mice. Cordycepin (15 mg/kg) was intraperitoneally injected at ZT11, as indicated by arrows. Yellow regions represent the light phase, and gray areas the dark phase. Sample sizes and *P* values from one-way ANOVA are included in the figure. Data are mean ± SD.

required for clock function. This observation is consistent with a recent publication that indicates that TIP60 may modify clock function through acetylation of BMAL1 (*32*, *33*). In contrast, another RUVBL-containing complex called the PAQosome (particle for arrangement of quaternary structure; also known as R2TP/PFD-like) complex (*34*, *35*), is apparently uninvolved with this regulation: Knocking down subunits of the PAQosome complex other than the *RUVBL* genes only resulted in modest, if any, circadian phenotypes (fig. S12). Together, these findings indicate that RUVBL2 may regulate the clock.

Our subsequent evaluation of interactions between RUVBL2 and core clock components found that RUVBL2 can be mutually coimmunoprecipitated with BMAL1 in mouse fibroblast cells (fig. S13, A and B) and that this interaction appears to require the N-terminal region of BMAL1 (fig. S13C). Notably, the recombinant RUVBL2 protein specifically pulled down BMAL1, suggesting a direct interaction (Fig. 3D). Further supporting this interaction, our ChIP-seq analysis revealed that most BMAL1-bound loci (65%) were co-occupied with RUVBL2 in U2OS cells (Fig. 3E). Moreover, time-course liver ChIP-seq analyses using antibodies against RUVBL2 and BMAL1 showed that, in vivo, both proteins bound to the E-box *cis*-elements of clock-controlled genes and both did so rhythmically and in the same phase (Fig. 3, F and G). Thus, RUVBL2, working with BMAL1, regulates clock-controlled gene expression and may be viewed as a clock modifier.

Clock-related structural and biochemical analyses of RUVBL2

Seeking to understand the detailed mechanism(s) through which RUVBL2 mediates cordycepin-induced clock phase shifting, we attempted to obtain a crystal structure of human RUVBL2 in complex with cordycepin. During experimental planning, we examined a previously reported RUVBL2 *apo* structure (*36*, *37*) and structures of RUVBL2 in complex with adenosine 5′-diphosphate (ADP) (*38*, *39*). These structures, considered alongside RUVBL2's known ATPase activity and cordycepin's close structural similarity to adenosine, suggested the possibility that RUVBL2 may bind with a phosphorylated and perhaps polyphosphorylated form of cordycepin. We did not obtain a cocrystal structure when purified RUVBL2 was incubated with cordycepin (2 mM), but we readily obtained a 2.50-Å structure of RUVBL2 in complex with cordycepin triphosphate (CoTP; see Fig. 4, A and B, and data file S6). The distribution of electron densities in the cocrystal structure obtained from incubation with CoTP also indicated binding of RUVBL2 with cordycepin diphosphate (CoDP) and with intermediate forms of Mg^{2+}-/water-bound CoDP (fig. S14). These findings are consistent with the known ATPase enzymatic activity of RUVBL2.

Our finding that a phosphorylated form of cordycepin is apparently the major ligand of RUVBL2 prompted us to reevaluate interactions between RUVBL2 and various cordycepin forms. We initially confirmed the presence of CoTP in U2OS cells via MS (fig. S15), and both microscale thermophoresis (Fig. 4C) (*40*) and SPR binding assays (fig. S16A) showed that the RUVBL2-CoTP interaction was more than 10-fold stronger than the RUVBL2-ATP interaction. Pull-down assays using purified proteins showed that CoTP and CoDP disrupted the interaction between RUVBL2 and BMAL1, whereas cordycepin itself, ATP, and ADP did not (Fig. 4D). Consistently, when an adenosine kinase inhibitor was coincubated with cordycepin, the phase shift effect was completely eliminated (Fig. 4E), suggesting that the phosphorylation of the cordycepin is required for its phase-shift effect. To further validate the relationship between cordycepin-induced phase shift and the CoTP-RUVBL2 interaction, we constructed a series of mutations around CoTP binding pocket in RUVBL2 protein to perturb the interaction and evaluated their impact on the cordycepin effect of the phase shift. We found that two RUVBL2 mutant variants, D299Q and A85T, increased more

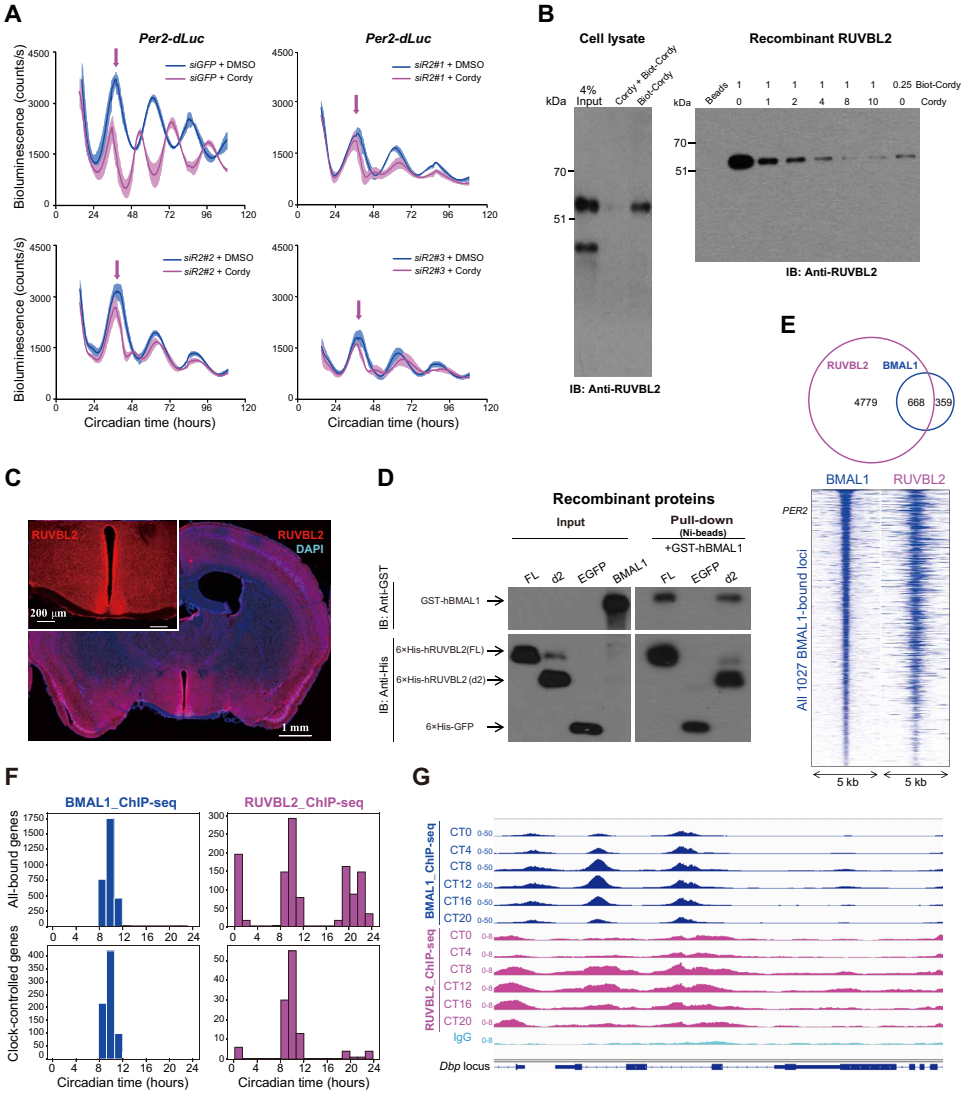

Fig. 3. Identification of RUVBL2 as the cordycepin target that mediates the phase shift. (**A**) Effects of individual siRNA molecules against *RUVBL2* or *GFP* on cordycepin-induced circadian phase shift in *Per2-dLuc* U2OS cells. For each curve, the data are mean ± SD ($n = 3$) and circadian time (CT) starts at the medium change. (**B**) Physical interaction between RUVBL2 and cordycepin was examined with biotinylated pull-down competition assays. Left, whole cell lysate; right, purified protein. (**C**) Immunofluorescence staining of the RUVBL2 protein in the mouse adult brain at CT12 ($n = 5$). Whole panel: Coronal section of the WT mouse brain staining with 4′,6-diamidino-2-phenylindole (DAPI) (blue) and an anti-RUVBL2 antibody (red); upper-left insert: zoom-in on the hypothalamus region. (**D**) Recombinant RUVBL2 pulled down with purified BMAL1 protein. Lanes shown in order from left to right: full-length of recombinant human RUVBL2 (FL); domain 2 deletion RUVBL2 variant (d2, lacking of the regulatory domain for the helicase activity); negative control enhanced green fluorescent protein (EGFP); glutathione *S*-transferase (GST)–tagged recombinant human BMAL1 protein; purified BMAL1 with RUVBL2 FL; purified BMAL1 with EGFP; and purified BMAL1 and the d2 RUVBL2 variant. (**E**) A Venn diagram (upper) and a heat map (lower) of ChIP-seq data showing the high rate of co-occupancy of chromatin loci (genome wide) by RUVBL2 and BMAL1 in U2OS cells. (**F**) Histograms from time-course ChIP-seq analyses of mouse liver. The analyzed liver clock-controlled genes (1444 genes in total) are from a previous report (*31*). (**G**) A UCSC (University of California, Santa Cruz) genome browser view of the liver *Dbp* locus showing the dynamic occupancies of BMAL1 and RUVBL2.

than 30-fold and decreased 100-fold the binding affinity compared with WT RUVBL2, respectively (fig. S16B). Because *RUVBL2* is an essential gene, we were not able to knock in the two mutants into U2OS cells. As an alternative, we ectopically expressed the D299Q variant of RUVBL2 that showed increased protein interaction with CoTP in U2OS cells, which caused a significant increase in the extent of the clock phase-shift effect ($P < 0.001$). Conversely, expression of the A85T variant that reduced the RUVBL2 interaction with CoTP decreased the phase-shift effect with a higher dose of cordycepin treatment in cells (Fig. 4, D and F, and fig. S16B).

Necessity of E-box gene transcriptional activation for phase shifts

We demonstrated that cordycepin treatment disrupted interactions between RUVBL2 and BMAL1 but had not yet considered the functional implications of this disruption on the transcriptional dynamics of known BMAL1-regulated E-box target genes. We therefore implemented a mathematical model based on mass action kinetics parameters from a previous theoretical study of circadian clock oscillation (*41*) with the aim of simulating how each core clock gene likely responds to the phase perturbation induced by cordycepin treatment. To model the effects of cordycepin on E-box gene transcription, we first simulated repression or induction effects on E-box clock genes (*PER1*, *PER2*, *DBP*, and *NR1D1*) at the exact peak of *PER2* expression; specifically, we simulated either transcriptional repression (80%) or induction (5×) in reference to an arbitrarily defined initial level, and then monitored the gradual equilibration of the repressed or induced systems. These perturbations showed a marked difference on the simulated circadian phase changes (Fig. 5A). We found that when the induction setting reached 3×, the *PER2* expression curve displayed a strong reversal (that is, a type 0–resetting phenotype). This modeled phase reversal only occurred when the perturbation representing cordycepin treatment was applied at the *PER2* peak, not at the *PER2* trough. Moreover, none of the repression simulations we conducted predicted any phase reversal in the E-box gene transcription, even at the 95% repression setting.

To experimentally corroborate the induction-based molecular mechanism through which cordycepin apparently alters the circadian phase in mice, we imaged luciferase activity in PER2::LUC knock-in mice. We found that a liver luciferase signal was induced within 1 hour of cordycepin treatment, supporting the hypothesis that such treatment induces PER2 expression in vivo (Fig. 5B). Moreover, quantitative

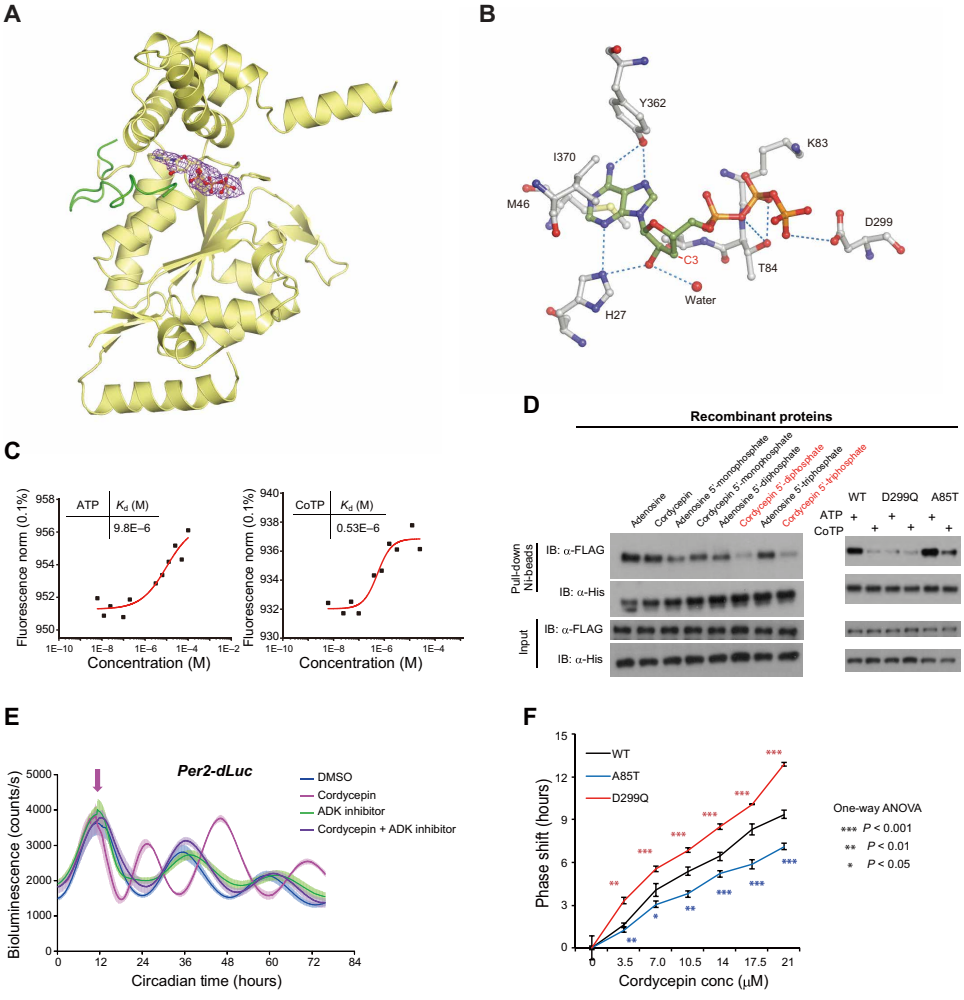

Fig. 4. Structural and biochemical analyses of RUVBL2 involvement in cordycepin phase-shift effects. (**A**) Cartoon presentation of RUVBL2 in complex with cordycepin 5′-triphosphate (CoTP). The green region representing the N-terminal segment of RUVBL2 (amino acids 23 to 41), which is otherwise flexible and hence invisible in the *apo* RUVBL2 crystal [Protein Data Bank (PDB) code: 6H7X], folds into the protein (light yellow) in the presence of CoTP (stick ball). In this figure, chain E of heterohexamer was used. The CoTP omitted electron density (weighted F_o-F_c, in purple blue) was countered at 2.0 σ. (**B**) Interactions between CoTP and the indicated amino acid residues of RUVBL2. Red C3 within the compound highlights the 3′-deoxylation site, which distinguishes cordycepin from the naïve adenosine. Dashed lines represent hydrogen bonds. (**C**) Microscale thermophoresis assay quantifying the RUVBL2-CoTP and RUVBL2-ATP interactions. Because the thermophoretic dynamics of fluorescent labeled RUVBL2 changes upon binding to a nonfluorescent ligand CoTP, resulting in different traces, the fluorescence of thermophoresis was normalized with two temperature stimulations (F norm). (**D**) Left: Effect of phosphorylated cordycepin on the interaction between the purified RUVBL2 (His-tagged) and BMAL1 (Flag-tagged) proteins. Right: Effect of D299Q mutant variant of RUVBL2 on RUVBL2 interaction with BMAL1. (**E**) Effect of adenosine kinase inhibitor (10 μM) on cordycepin (25 μM)–induced phase shift in *Per2-dLuc* U2OS cells ($n = 4$, CT0 is at 24 hours post-medium change). Data are presented as mean ± SD. (**F**) Effects of cordycepin on the circadian phases shift of *Per2-dLuc* U2OS cells ectopically expressing D299Q, A85T, or WT variant of RUVBL2 ($n = 4$). Data are presented as mean ± SD.

polymerase chain reaction (qPCR) analysis of nine core clock genes in the livers of cordycepin-treated mice showed that cordycepin treatment up-regulated the expression of the four known E-box containing clock genes (*Per1*, *Per2*, *Dbp*, and *Nr1d1*) but did not affect or down-regulate the expression of clock genes without E-box elements (fig. S17). This cordycepin-mediated induction of E-box gene expression was observed in qPCR assays specifically targeting nascent mRNA, highlighting the rapid effects of this compound. Another agreement between the model and our experimental results relates to *BMAL1* expression. The model predicted no phase change in the first 12 hours for *BMAL1* expression, and consistent with this, our luciferase reporter experiments showed that *Bmal1-dLuc* remained "on course" with the clock for the first 12 hours after drug treatment. The *BMAL1* curve subsequently appeared, representing a strong forward push of the phase that manifested as a 12-hour reversion (Fig. 5, C and D). These modeling and in vivo results support that cordycepin directly affects the clockwork itself by inducing the transcription of E-box containing clock genes, ultimately regulating the circadian phase.

Regulation of the circadian clock phase by a megadalton super-complex

Recently, a single-particle electron microscopy (EM) study identified a megadalton transcriptional repressive super-complex that appears to inhibit the expression of clock genes and clock-controlled genes in a rhythmic fashion (*42*). To assess the potential impact of cordycepin on this super-complex, we conducted experiments using rhythmic *Bmal1*$^{-/-}$ fibroblasts expressing a Flag-hemagglutinin (HA) dual-tagged variant of BMAL1 (fig. S13). After tandem affinity purification of nuclear extracts from untreated cells, blue-native polyacrylamide gel electrophoresis (BN-PAGE) analysis revealed a super-complex of a size consistent with the previously reported PER-containing complexes (~1.4 MDa) (*42*). Following gel excision, MS-based proteomics analysis of the super-complex components identified three groups of potentially clock-related proteins: well-known core clock proteins (for example, BMAL1, CLOCK, PER, CRY, and CK1δ) (*43*), previously reported clock-associated proteins [including CIPC (*44*), DDX5 (*45*), THRAP3 (*46*), and PML (*47*)], and putative clock regulatory proteins like RUVBL2 and known RUVBL2-associated TIP60 complex components such as TRRAP, EP400, and RUVBL1 (Fig. 6A and data file S5) (*48*). These results, combined with our previous demonstration that knockdown of *TRRAP*, *EP400*, and other subunit genes altered the clock phase, amplitude, or period, indicate that the TIP60 complex appears to be engaged in clock regulation. In contrast, but consistent with our speculation that the PAQosome complex may not be involved in circadian regulation, we did not observe engagement of any PAQosome subunits other than RUVBL1/2 in this megadalton complex, either in our MS (data file S5) or immunoblotting results for native or denatured PAGE mobility assays (fig. S18).

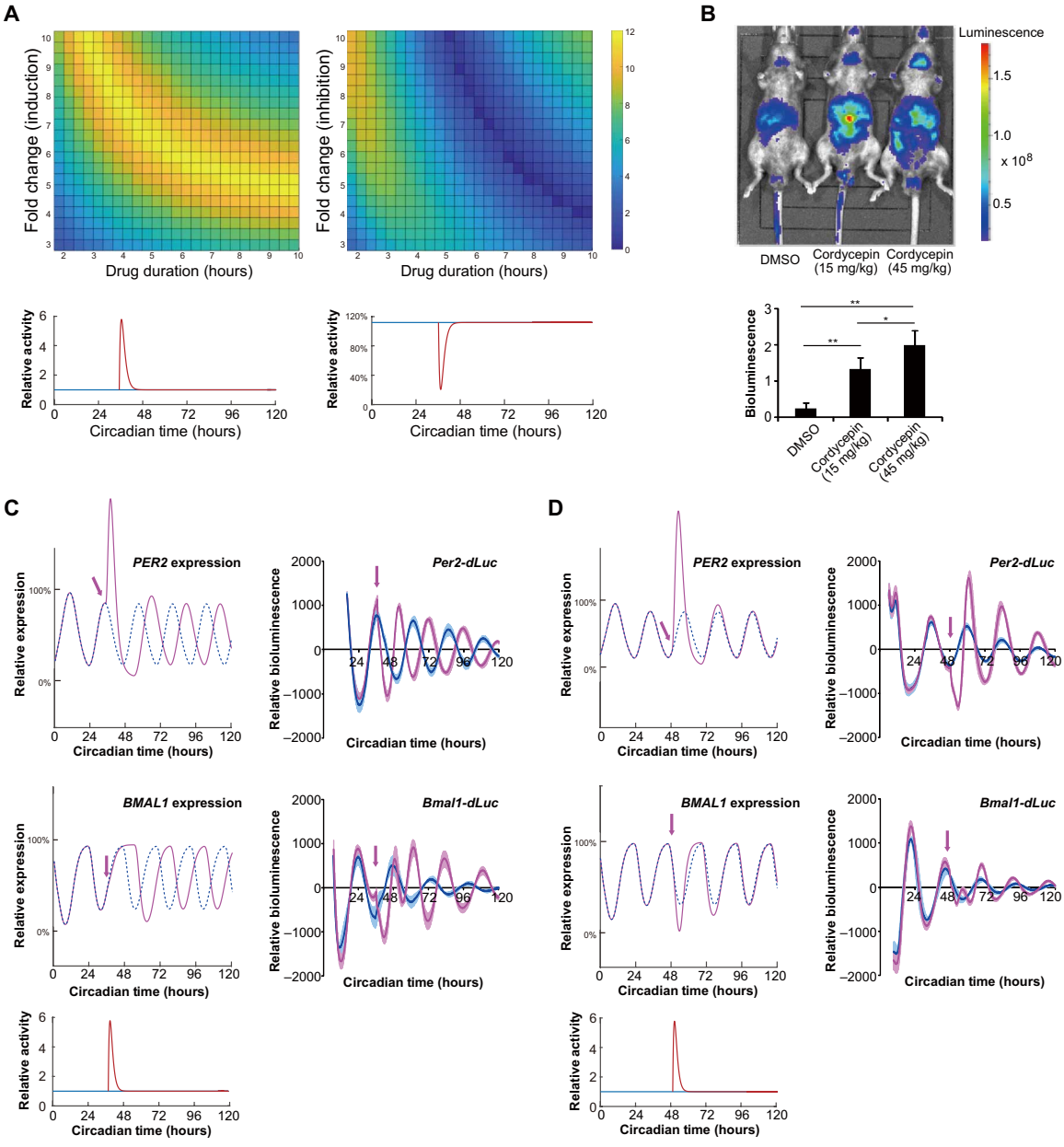

Fig. 5. Mass action kinetics modeling and experimental validation of cordycepin phase effects on E-box gene transcription. (**A**) Top: A mathematical simulation exploring relationships among phase-shift efficiency (heat map color bar), a compound's induction or inhibitory effect on E-box gene transcription (*y* axis), and varying drug exposure durations (pharmacokinetics) of the compound (*x* axis), assuming either a net inductive (left) or net inhibitory (right) influence. Bottom: Proposed changes of the transcription strength for E-box genes corresponding to the net inductive or net inhibitory impact of a compound (transcription activity under the untreated condition is set as "1" or 100%). (**B**) Live imaging of PER2::LUC mice administered cordycepin by intraperitoneal injection at ZT2. Data are presented as mean ± SD ($n = 5$ per group; $^*P < 0.05$ and $^{**}P < 0.001$, one-way ANOVA). (**C** and **D**) Direct comparison of theoretical trajectories and experimental results of *PER2* and *BMAL1* gene expression upon cordycepin treatment administered at either the peak or the trough (D) of the *Per2-dLuc* signal intensity. For the mathematical simulation, the maximal gene expression under the untreated condition is set as 100%. Dashed blue lines indicate the free-run controls, and solid pink lines stand for the trajectories altered by the perturbations. Data are presented as mean ± SD from triplicate samples ($n = 3$).

We note that cordycepin treatment caused disassembly of the repressive super-complex (Fig. 6B). We used cordycepin as a chemical probe to disrupt protein-protein interactions among the super-complex's components, including a previously reported clock interaction (BMAL1-CRY1) (*43*, *49*) and the RUVBL2-BMAL1 interactions we present here (Fig. 6C and fig. S19). These findings raise the possibility that RUVBL2 may function as a biomolecular nexus that connects multiple components of a clock super-complex comprising (at least) BMAL1 and CRY1 and support that the phase-shifting effect of cordycepin results from its disassembly of this clock super-complex through its targeting of RUVBL2.

To further demonstrate that cordycepin treatment affects the chromatin association of super-complex components, we performed

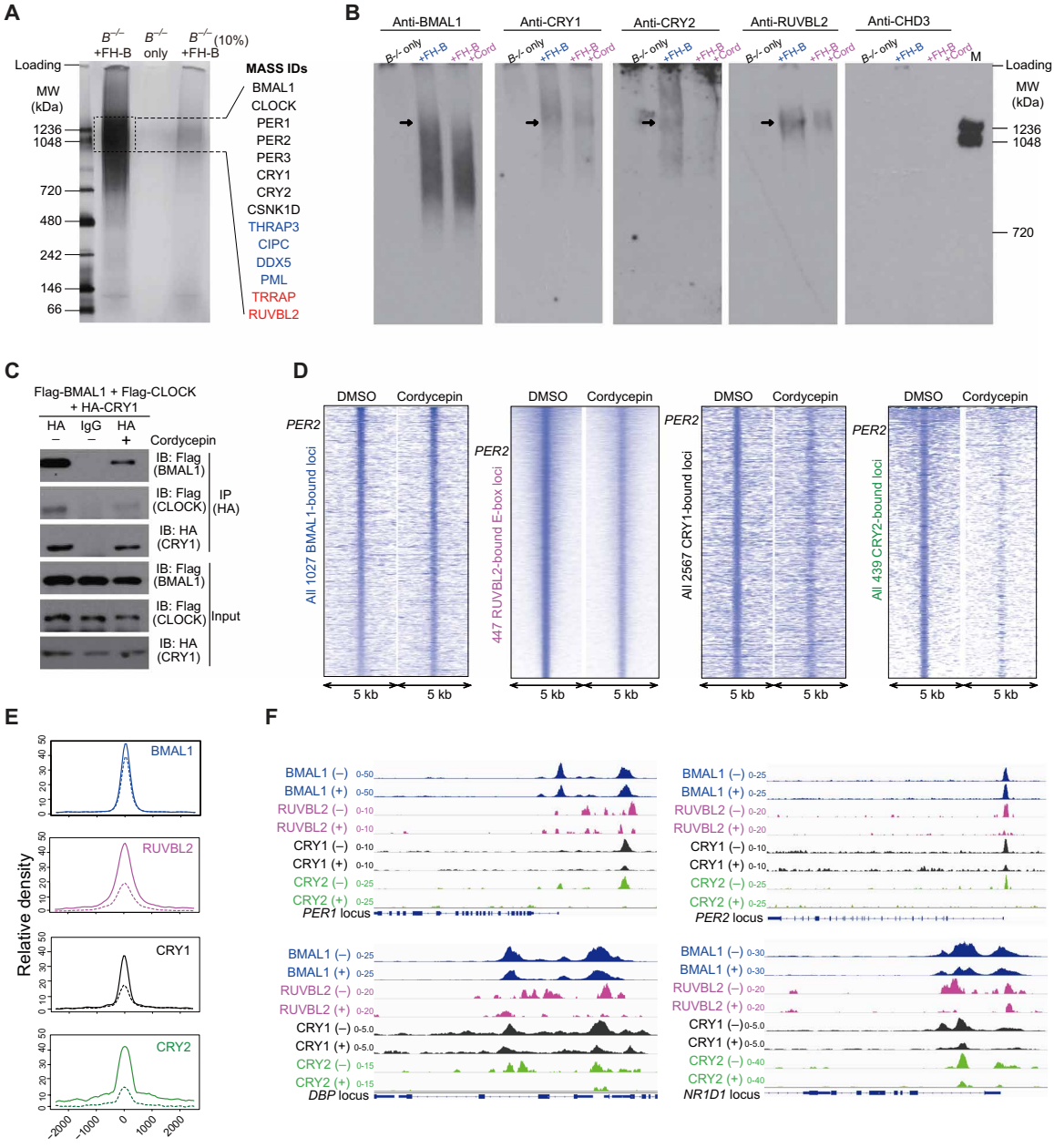

Fig. 6. An RUVBL2-containing super-complex regulates the circadian phase. (**A**) A megadalton circadian super-complex was detected by tandem purification with anti-Flag and anti-HA antibodies, elution of the complex from beads, BN-PAGE, and silver staining. The band containing the complex (size ~1.4 MDa) was subsequently excised (dash-box) and digested with trypsin and analyzed via nano–liquid chromatography–MS/MS (nanoLC-MS/MS). Lanes from left to right: *Flag-HA-Bmal1*–rescued *Bmal1*$^{-/-}$ cells ($B^{-/-}$ + FH-B); *Bmal1*$^{-/-}$ only ($B^{-/-}$ only) cells as a negative control; and 10× dilution of the left lane amount. Color coding for the identified proteins: black, known core clock components; blue, previously reported clock-associated proteins; red, RUVBL2 or other putative clock-associated proteins in this circadian megadalton complex. (**B**) Effect of cordycepin treatment on the megadalton circadian repressive super-complex was analyzed with BN-PAGE and Western blot, with samples tandem purified from nuclear lysates of unsynchronized cells: *Bmal1*$^{-/-}$ only ($B^{-/-}$ only); *Flag-HA-Bmal1*–rescued (+FH-B); and *Flag-HA-Bmal1*–rescued cells treated with 25 μM cordycepin for 1 hour (+FH-B + Cordy). Another DNA binding helicase (CHD3) served here as a negative control. Arrows indicate the BMAL1-containing ~1.4-MDa super-complex. (**C**) Co-IP of HA-CRY1 with FLAG-BMAL1/FLAG-CLOCK transiently expressed in HEK293T cells, with either DMSO or cordycepin treatment. (**D**) Heat map views of spike-in ChIP-seq data showing the chromatin-bound intensities of BMAL1, RUVBL2, CRY1, and CRY2, with either DMSO or cordycepin treatment in unsynchronized U2OS cells. (**E**) The top 400 binding intensities of E-box loci for each protein were further quantified. (**F**) UCSC genome browser views of the binding of BMAL1, RUVBL2, CRY1, and CRY2 with (+) or without (–) cordycepin treatment. The representative E-box–containing genes shown here are *PER1*, *PER2*, *DBP*, and *NR1D1*.

"spike-in" ChIP-seq in U2OS cells (*50*). We found that cordycepin treatment disrupted the interaction between RUVBL2 and BMAL1 (fig. S19), thereby repelling RUVBL2 from chromatin, whereas BMAL1 remained on chromatin following cordycepin treatment (Fig. 6, D to F). This ChIP-seq analysis of the changing chromatin landscape also showed that 1 hour of cordycepin treatment reduced the association of the known clock repressors CRY1 and CRY2 with chromatin (Fig. 6, D to F).

DISCUSSION

We conceived this cell-based screen to investigate the sensitivity of the circadian phase to various environmental stimuli. The use of two separate reporter lines and stringent screening criteria for potential regulators of the clock phase turned out to be key differentiators of our screen compared to previous efforts in this area. We observed multiple "type 1" phase shifters (defined as <6-hour shift) (*23*) that may be caused by the range of cell culture perturbations, such as pH and temperature. However, only the adenosine analogs identified and examined in our present study showed the "type 0" phase-shifting phenotype, underlying its mechanistic specificity.

Together, our results support a model wherein E-box genes are repressed by a circadian regulatory super-complex comprising the previously known clock complex (CLOCK/BMAL1 and PER/CRY) and an RUVBL2-containing complex. We show that when cordycepin interferes with RUVBL2, this super-complex disassembles, and inhibitory CRY proteins leave the chromatin (whereas CLOCK/BMAL1 remains on the chromatin); the lack of the CRY proteins relieves the repression, and E-box genes are transcribed. Subsequently, as the super-complex begins to reassemble, a new clock oscillation starts (fig. S20). Considering that it can be used to disrupt the super-complex and the clock period over a short timescale, cordycepin is well situated to be a powerful tool for future basic studies seeking to identify the various subunits of the modular subcomplexes that together form the megadalton transcriptional repressor super-complex.

Another notable aspect from comparing the *apo* structure and our cocrystal structure was that the N-terminal region of RUVBL2 (amino acids 23 to 41) undergoes a conformational change inwards from the main domain upon binding with CoTP (*38*). This change provides a plausible explanation for the dissociation of the interaction of RUVBL2 with other proteins like BMAL1, and this observation is conceptually similar to a recently reported finding from a cryo-EM study that binding with nucleotides may affect interactions between RUVBL2 and other binding partner proteins such as PIH1D1 (*39*). We speculated that a more flexible N terminus of RUVBL2 facilitates its association with BMAL1. Biochemically, the interaction of RUVBL2 with CoTP was ~20-fold stronger than with native ATP. This stronger interaction makes the N terminus–containing RUVBL2-CoTP complex a more intact structural entity, and hence, CoTP is an efficient disruptor of RUVBL2-BMAL1 interaction. Although CoTP lacks the hydrogen bond between the C3 hydroxyl group and the backbone carbonyl of Ala^{24} of RUVBL2, CoTP appears to have a lower dissociation free energy cost than ATP because of the absence of this C3 hydroxyl group. Thus, CoTP possibly has a net favorable free energy gain in binding RUVBL2 rather than ATP. Whether the intracellular ATP oscillation can affect this process needs to be investigated in the future.

Cordycepin and related compounds developed as probes will also help to characterize the timing and sequence of megacomplex assembly and disassembly, enabling the empirical delineation of (at minimum) a "repression stage" and a "derepression stage" for E-box–mediated transcriptional control of circadian rhythms in cells. Previous clock-modifying compounds have been unable to penetrate the BBB, rendering them unsuitable for behavioral assays unless cumbersome and invasive intracerebro-ventricular injection is used (*27*). The ability of cordycepin to penetrate the BBB, its rapid onset effect, and its 12-hour phase reversal phenotype make it appear as a potential candidate as a drug to treat jet lag. Looking beyond treatment of acute circadian clock disorders, one can envision that the ability to reliably therapeutically entrain rhythmicity could help treat a broad swath of known clock-related chronic disorders as well.

However, we are also aware that there are limitations to our research. First, our in vivo studies were all based on rodents. It is important to note that nocturnal animals such as mice and rats have opposite patterns of behavior and clock gene expressions as diurnal ones like primates (*22*). One cannot directly extrapolate the dosing time points from mice to humans. Second, the relatively low potency of cordycepin requires high dosing to the animals to obtain the behavioral effect. It is thus desirable to develop analogs for better pharmacokinetic and pharmacodynamic properties, as well as testing in human cells, before going to clinical examinations.

MATERIALS AND METHODS

Study design

The aim of this study was to identify potential small-molecule compounds that facilitate fast shifting of the clock phase in mammals and to study the molecular mechanism underlying the phase regulation. We began the study with a cell-based chemical screen and identified a set of adenosine analogs including cordycepin that can quickly shift the clock phase in human cells. We subsequently verified these compounds using a locomotor activity assay in mice. To characterize functional target(s) of cordycepin, we conducted a cellular genetic screen coupled with a MS-based approach, and identified RUVBL2, an AAA-type ATPase, as a clock phase modifier. We used further structural and biochemical analyses to investigate how the physiological metabolite of cordycepin binds to RUVBL2 to reset the clock phase. All mice were maintained in specific pathogen–free (SPF) environment with food and water ad libitum under a 12-hour: 12-hour light-dark (LD) photoperiod unless specifically noted. No animals were excluded in this study. All procedures in this protocol were conducted with the approval of the Institutional Animal Care and Use Committee of the National Institute of Biological Sciences, Beijing, in accordance with the governmental regulations of China. Additional methods are available in the Supplementary Materials.

Mouse locomotor assay

WT C57BL/6J mice at the age of 2 to 5 months old were entrained under a 12-hour light (~50 lux light intensity)/12-hour dark cycle at 25°C for 2 weeks. One week after mouse clock synchronization to the ambient light/dark cycle, cordycepin or dimethyl sulfoxide (DMSO) was dissolved in saline and intraperitoneally injected (15 mg/kg) into mice 1 hour before light off (ZT11) or light on (ZT23). Light/dark cycles were subsequently phase advanced or delayed by 8 hours. Cordycepin or DMSO was injected again 24 hours later. After 2 weeks of continuous recording, locomotor activity was analyzed with ClockLab software (Actimetrics) following the procedures described previously (*27*).

Statistical analysis

In all experiments, error bars represent SD unless otherwise noted. Statistical significance was determined using unpaired two-sided Student's *t* tests when only two groups were analyzed and data were normal. One-way analysis of variance (ANOVA) was used when there were more than two groups being analyzed. $P < 0.05$ was considered statistically significant. $n \geq 3$ samples for each group, unless specifically noted.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Chemical screen for clock shifter compounds.
Fig. S2. BioDare2 analyses of the various nucleotides including cordycepin for the clock effect.
Fig. S3. Cordycepin hijacks the adenosine metabolism pathways to shift circadian phase.
Fig. S4. Cordycepin regulates the clock phase in the tissue explants.
Fig. S5. Cordycepin penetrates through the BBB but degrades quickly in the rodent brain.
Fig. S6. The phase shift of cordycepin in mouse locomotor depends on the time of its administration.
Fig. S7. Combined approaches reveal RUVBL2 as the target of cordycepin in regulating the clock phase.
Fig. S8. Knockdown efficiency of *RUVBL2* in U2OS cells.
Fig. S9. Rhythmic expression of RUVBL2 in the SCN.
Fig. S10. Two mouse *Ruvbl* genes are rhythmically expressed in vivo.
Fig. S11. TIP60 complex is likely involved in the clock regulation.
Fig. S12. The PAQosome complex may not be involved in the clock regulation.
Fig. S13. Cell lines and the BMAL1 coimmunoprecipitation assays.
Fig. S14. Structural analysis of RUVBL ATPases and phosphorylated cordycepin.
Fig. S15. Pharmacokinetic analyses of cordycepin and CoTP in U2OS cells.
Fig. S16. Physical interactions between RUVBL2 proteins and CoTP/ATP.
Fig. S17. Cordycepin induces the expression of E-box genes in vivo.
Fig. S18. The PAQosome complex may not be present in the circadian super-complex.
Fig. S19. Cordycepin disrupts the interaction between RUVBL2 and BMAL1.
Fig. S20. Cartoon of the proposed megadalton circadian repressive super-complex on an E-box containing clock gene.
Data file S1. Phase responsive curve of the cordycepin treatment analyzed by BioDare2.
Data file S2. Cotreatment of pentostatin and cordycepin analyzed by BioDare2.
Data file S3. siRNA screen targets responsible for the cordycepin treatment effect.
Data file S4. IP-MS to identify the biotinylated cordycepin-bound proteins.
Data file S5. IP-MS to identify the megadalton super-complex components via BN-PAGE.
Data file S6. Statistics of crystallization data collection and structure refinement.
Data file S7. Primers used for RT-PCR/qPCR.
Data file S8. All digital data used for generating figures.
References (*51*–*62*)

View/request a protocol for this paper from *Bio-protocol*.

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Chemical perturbations reveal that RUVBL2 regulates the circadian phase in mammals

Dapeng Ju, Wei Zhang, Jiawei Yan, Haijiao Zhao, Wei Li, Jiawen Wang, Meimei Liao, Zhancong Xu, Zhiqiang Wang, Guanshen Zhou, Long Mei, Nannan Hou, Shuhua Ying, Tao Cai, She Chen, Xiaowen Xie, Luhua Lai, Chao Tang, Noheon Park, Joseph S. Takahashi, Niu Huang, Xiangbing Qi and Eric Erquan Zhang

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Shifting clock gears

Molecular circadian oscillators help control sleep/wake periodicity. Ju *et al.* show that an AAA-type ATPase RUVBL2 (RuvB-like 2) is part of a molecular mechanism that regulates the circadian clock. They found that the adenosine derivative cordycepin shifted the circadian phase by 12 hours in human cells in vitro and mouse cells ex vivo and accelerated clock acclimation in a mouse model of jet lag. Mechanistically, cordycepin acted to modify the clock by preventing the interaction of RUVBL2 and clock gene BMAL1, resulting in the dissociation of a repressive complex at specific chromatin loci and the derepression of clock gene transcription.

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