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Dissecting role of AAA-ATPase RUVBLs in circadian system via mathematical modeling

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The circadian genes in mammals are involved in a transcription-translation feedback loop, whereas in cyanobacteria, there exists a post-translational oscillator (PTO) consisting of KaiA, KaiB, and KaiC. KaiC has both ATPase and kinase activities. ATP binding, hydrolysis and phosphate transfer drive the dynamics of circadian system. In this study, we built a mathematical model for our latest found circadian genes *Ruvbl*1/2 (*Ruvbls*) and added the model to a previously published model for mammalian circadian system. RUVBLs are the first ATPase known to directly participate in the mammalian circadian system. We described the entire process whereby an ATPase is involved in the traditional transcription-translation feedback loop. Additionally, a phase-shift adenosine-like molecule, cordycepin, which was discovered from our high-throughput screening and targeted RUVBLs, was also simulated in our mathematical model. Notably, this model gives a more detailed description of circadian system, and considers the participation of ATPase for the first time, which can lead to a deeper insight into mammalian circadian clock regulation. Our model aligns well our experimental data. Further, based on wet-lab experiments and dry-lab modeling, we discussed the role of ATP and ATPase in mammalian circadian system. Finally, we compared the similarities and differences between KaiC and RUVBLs, and discussed the potential of RUVBLs as a component of mammalian post-translational oscillator.

1. Introduction

From cyanobacteria to mammals, circadian clock exists in almost all organisms on Earth, which is intrinsic and autonomous, and can be entrained

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by the external environment [1]. The fundamental mechanism of the circadian clock in mammals is transcription-translation feedback loop (TTFL). A triple interlocking loop was proposed to describe this feedback. The core loop is composed of the E-box genes, *Per* and *Cry*, whose can be promoted by circadian activator CLOCK and BMAL1. With the accumulation of PER and CRY, they act as repressor and bind to CLOCK-BMAL1, ultimately inhibiting the transcription of themselves. Similar transcription-translation feedback of transcription activators and repressors also occurs in the ROR-REV loop and the DBP-E4BP4 loop, which with the PER-CRY loop together, generate a robust, periodic, cosine-like oscillation [2, 3]. Consequently, a circadian rhythm can be described with three indicators: period (24 h under light-dark cycle and a little bit shorter or longer intrinsically under free-running conditions in the majority of mammals), amplitude (indicating the robustness of circadian rhythm), and phase.

Unlike mammals, one of the earliest organisms in the world, cyanobacteria, possess a post-translational oscillator (PTO), which is successfully reconstituted *in vitro* [4, 5, 6]. Post-translational modification refers to the covalent and generally enzymatic modification of proteins following protein biosynthesis, and PTO is the circadian oscillator based on the control by sequences of self-phosphorylation and dephosphorylation of the ATPase and kinase, KaiC [28]. This post-translational oscillator is composed simply of three proteins, KaiA, KaiB, and KaiC. KaiC can be divided into two structurally similar domains CI and CII, both of which can bind ATP. CI has ATPase activity, while CII has kinase activity. In the presence of ATP, KaiA, and KaiB, the phosphorylation and dephosphorylation of KaiC can oscillate automatically within a period of about 24 h, and protein binding, conformation change, etc., also undergo a perfect oscillation [4, 5, 6]. Although conformational changes and binding patterns have been well studied through biochemical and structural biological approaches, the basic generation of this oscillation remains as different hypotheses. A common sight of these hypotheses is to regard CI and CII domain of KaiC as a time keeper and an energy donor of the Kai system, no matter they call it physical/chemical oscillator or driver/timer [7]. And thus, the aggregation and dissociation of these proteins, the ATPase and kinase activity, are all regulation factors of PTO.

Although the post-translational oscillator has never been found in mammals, and the widely accepted core clock proteins in mammals are transcription factors, the possibility of its existence cannot be denied. In previous study, we have identified an AAA-ATPase, RUVBL2, as a novel circadian clock component. RUVBL2 was demonstrated to form a super-complex with

circadian transcription factors, and ChIP-seq showed a colocalization of RUVBL2 and BMAL1, CRY1, and CRY2 at E-box motif of some clock genes [8]. BMAL1, CRY1, and CRY2 were also found to bind to *Ruvbl*2 promoter region in another study [1], indicating that *Ruvbl*2 expression may also be regulated in a circadian manner. RUVBL2 is not a transcription factor, and the participation of RUVBL2 in the core circadian circuit indicates that ATP and ATPase may also play a role in the circadian system (its homolog RUVBL1 also has ATPase activity, and since RUVBL1 always form a hetero-hexamer with RUVBL2, we call RUVBL1/2 by a joint name RUVBLs in this paper). We found that cordycepin, an ATP-like small molecule, can bind to RUVBLs pocket after phosphorylated to cordycepin-triple-phosphate (CoTP), and has the ability to shift phase dramatically [8]. Although the post-translational regulation of RUVBLs has not been fully established experimentally, and the mechanism of cordycepin phase-shift effect is not easy to study at atomic level, we are actively attempting to identify the similarities between RUVBLs and Kai system, and the possibility for RUVBLs to act as a post-translational oscillator of the circadian clock.

It is easy to use differential equations to describe the transcription-translation feedback loop, and various accurate mathematical models are available for the mammalian circadian system. Therefore, we intended to use mathematical model to establish the new circadian circuit including RUVBLs, and provide a deeper insight into the participation of RUVBLs that is difficult to observe in wet experiments. In this study, we added the new circadian component, RUVBLs, to the detailed model of mammalian circadian clock published by Jae Kyoung Kim and Daniel B Forger [9]. As the differences between RUVBL1 and RUVBL2 are not well studied, we treated the two ATPases as the same variable. This model predicted the feedback loop of *Ruvbls* mRNA, RUVBLs monomer, RUVBLs hexamer, super-complex, and then to mRNA again. ATP hydrolysis was set as a unilateral switch of the RUVBLs loop, and through parameter screening, we established the relationship between ATPase activity and circadian period and robustness. Cordycepin was also simulated in this model, and the putative working mechanism in this model also had a phase shift effect. Furthermore, we compared the RUVBLs mammalian model with Kai system model of cyanobacteria [7, 10], and discussed similarities and differences between the two systems.

2. Results

2.1. Mathematical modeling of mammalian circadian system including the new clock gene *Ruvbls*

We established this model based on a published detailed model for the mammalian circadian clock [9], and add RUVBLs to the model (Figure 1(a)).

In this model, RUVBLs monomers can aggregate to hexamer, bind, and hydrolyze ATP. After the hydrolysis product ADP dissociates from RUVBLs hexamer, it can bind with CLOCK/BMAL/CRYs and form a super-complex with transcription inhibition activity to inhibit the expression of E-box genes and itself. The processes of transcription, translation, E-box activation and inhibition, and RNA and protein degradation are all modeled in the same way as the previous model. New variables and related model of biological processes are described as follows.

2.1.1. mRNA level of *Ruvbls* Although *Ruvbls* have not been defined as E-box genes yet, previous studies revealed the binding of BMAL1, CRY1, and CRY2 on *Ruvbl*2 promoter [8]. Therefore, *Ruvbls* are classified into the same group as *Per*1, *Per*2, and *Cry*1 and share the same parameters related to transcription.

Similar to other mRNA concentrations of circadian genes,

$$\text{DNA} \xrightarrow{tr-factor} \text{mRNA}_{nuc} \tag{1}$$

$$\text{mRNA}_{nuc} \xrightarrow{k_f} \text{mRNA}_{cyt} \tag{2}$$

$$\text{mRNA}_{nuc} \xrightarrow{k_d} decomp. \tag{3}$$

$$\text{mRNA}_{cyt} \xrightarrow{k_d} decomp. \tag{4}$$

Where $tr-factor$ represents transcription factor, *nuc* represents nucleus, *cyt* represents cytoplasm, k_f is the rate for folding and nuclear export, k_d represents the degradation rate, and *decomp* represents degradation of the reactant. Because the concentration of DNA in cell was regarded as a constant, we could include it in the transcription rate constant. If the E-box activation probability was $F[\lambda(t)]$, and assume the mRNA folding, transfer,

and degradation process obey the law of mass action, so the change rate of mRNA_{nuc} is:

$$\frac{d[\mathrm{mRNA}_{nuc}]}{dt} = tr \cdot F[\lambda(t)] - k_f \cdot [\mathrm{mRNA}_{nuc}] - k_d \cdot [\mathrm{mRNA}_{nuc}] \tag{5}$$

And the difference of mRNA in the cytoplasm follows:

$$\frac{d[\mathrm{mRNA}_{cyt}]}{dt} = k_f \cdot [\mathrm{mRNA}_{nuc}] - k_d \cdot [\mathrm{mRNA}_{cyt}] \tag{6}$$

2.1.2. RUVBLs monomer *Ruvbls* mRNA is translated to a monomer protein with the translation rate tl:

$$\mathrm{mRNA}_{cyt} \xrightarrow{tl} \mathrm{RUVBL}_{mono} \tag{7}$$

Degradation of the protein is only considered in the monomer state with the degradation rate k_{deg}:

$$\mathrm{RUVBL}_{mono} \xrightarrow{k_{deg}} decomp. \tag{8}$$

Monomer can aggregate to form an *apo* hexamer, so there is a chemical equilibrium:

$$6\mathrm{RUVBL}_{mono} \rightleftharpoons \mathrm{RUVBL}_{hex}^{apo} \tag{9}$$

Considering the processes above, and assuming that the aggregation of RUVBL monomer is an elementary reaction, the concentration of RUVBLs monomer follows the ordinary differential equation [30]:

$$\begin{aligned} \frac{d[\mathrm{RUVBL}_{mono}]}{dt} =& tl \cdot [\mathrm{mRNA}_{cyt}] - k_{deg} \cdot [\mathrm{RUVBL}_{mono}], \\ & - k_{agg} \cdot [\mathrm{RUVBL}_{mono}]^6 + k_{dis} \cdot [\mathrm{RUVBL}_{hex}^{apo}] \end{aligned} \tag{10}$$

Where k_{agg}/k_{dis} represent the aggregation and dissociation rate of the hexamer, respectively.

2.1.3. *apo* RUVBLs hexamer Only the *apo* hexamer has the ability to bind ATP, which is reversible, and protein degradation is not considered in the *apo* hexamer state. The chemical equilibrium can be described as:

$$\mathrm{RUVBL}_{hex}^{apo} \rightleftharpoons \mathrm{RUVBL}_{hex}^{\mathrm{ATP}} \tag{11}$$

2.1.4. RUVBLs hexamer with ATP or ADP In this system, there is no ATP synthetase, and ATP hydrolysis is not reversible; ATP hydrolysis is the only one-directional switch of this feedback loop:

$$\text{RUVBL}_{hex}^{\text{ATP}} \xrightarrow{k_{hydro}} \text{RUVBL}_{hex}^{\text{ADP}} \tag{12}$$

Where k_{hydro} represents the hydrolysis rate for RUVBLs, which indicates the ATPase activity. We assumed that the only source of ADP is the hydrolysis of ATP, and the only functional RUVBLs-ADP complex comes from RUVBLs-ATP complex. Consequently, the natural binding of released ADP on *apo* RUVBLs is not included in this math model.

2.1.5. *apo* RUVBLs hexamer after ATP hydrolysis, a high-energy conformation After ATP hydrolysis and ADP dissociation, we supposed a transient state with energy to be the only state of RUVBLs that can form a super-complex with CLOCK/BMAL1/CRYs, which can also depolymerize to monomer. When dissociated from the super-complex, the high energy transient state will disappear (for the energy is consumed in formation of the super-complex), consequently, the hexamer returns to the initial *apo* hexamer (Figure 1(b)).

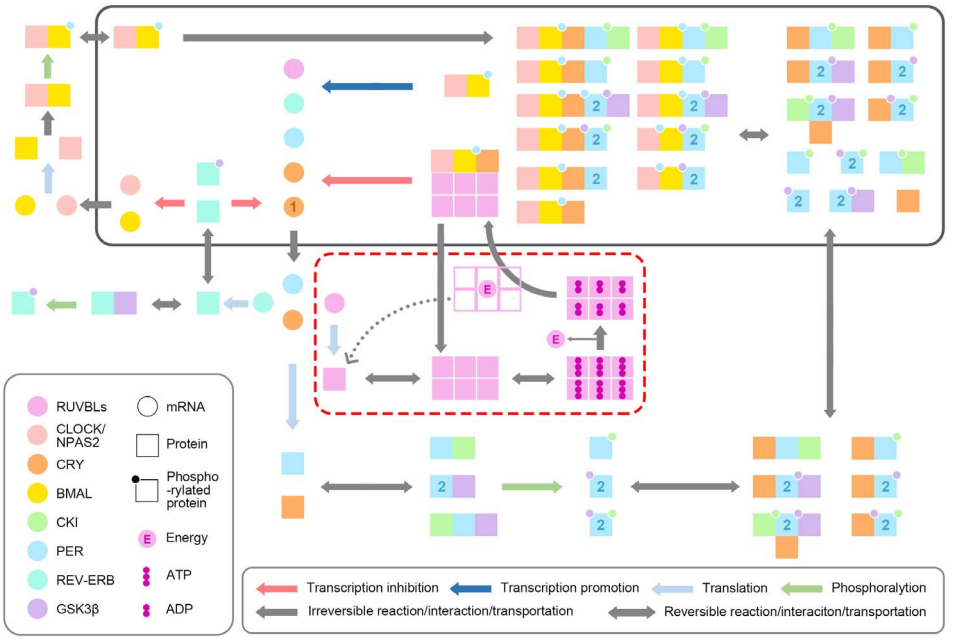
2.1.6. Super-complex composed of RUVBLs and CLOCK/BMAL1/CRYs Our previous ChIP-seq data indicated that RUVBLs colocalize with BMAL1, CRY1, and CRY2 at E-box motifs, and that RUVBLs can be co-immune precipitated with these three proteins [8], so in this model, we propose that the high-energy transient state of apo RUVBL2 hexamer binds to CLOCK/BMAL1/CRYs complex, and in this case, the transcription repressor of E-box will be the complex including RUVBLs.

These processes can be dissected as the following reactions:

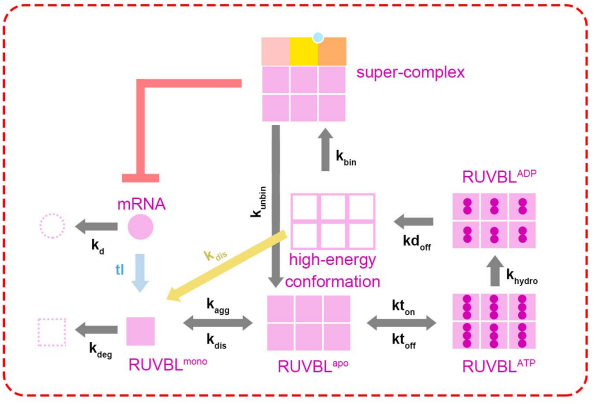
$$\text{RUVBL}_{hex}^{\text{ADP}} \xrightarrow{k_{off}} \text{RUVBL}_{hex}^{apo,E} \tag{13}$$

$$\text{RUVBL}_{hex}^{apo,E} + \text{CLOCK/BMAL/CRYs} \rightleftharpoons \text{super} - \text{complex} \tag{14}$$

ATP concentration is set to be abundant and constant, which means that there is no ATP/ADP ratio, and the effects of concentration on ATP/ADP binding rate are not concerned in this model. Parameters $kt_{on}/kt_{off}/kd_{on}/kd_{off}$ in this model are actually the real K_{on}/K_{off} with the concentration effect of ATP/ADP.



(a) Detailed model of mammalian circadian clock, adapted from [9]



(b) RUVBLs feedback loop

Figure 1: Schematic of the detailed mammalian circadian clock model.

Considering all processes together, the change rate of each state of RUVBLs hexamers can be described in the following differential equations:

$$\begin{aligned}\frac{d[\mathrm{RUVBL}_{hex}^{apo}]}{dt} =& k_{agg} \cdot [\mathrm{RUVBL}_{mono}]^6 - k_{dis} \cdot [\mathrm{RUVBL}_{hex}^{apo}], \\ & - kt_{on} \cdot [\mathrm{RUVBL}_{hex}^{apo}] + kt_{off} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}], \\ & + k_{unbin} \cdot [\mathrm{super-complex}]\end{aligned} \tag{15}$$

$$\begin{aligned}\frac{d[\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}]}{dt} =& kt_{on} \cdot [\mathrm{RUVBL}_{hex}^{apo}] - kt_{off} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}], \\ & - k_{hydro} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}]\end{aligned} \tag{16}$$

$$\frac{d[\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}]}{dt} = k_{hydro} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}] - kd_{off} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}] \tag{17}$$

$$\begin{aligned}\frac{d[\mathrm{RUVBL}_{hex}^{apo,E}]}{dt} =& kd_{off} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}] - k_{dis} \cdot [\mathrm{RUVBL}_{hex}^{apo,E}], \\ & - k_{bin} \cdot Nf \cdot [\mathrm{RUVBL}_{hex}^{apo,E}] \cdot [\mathrm{CLOCK/BMAL/CRYs}]\end{aligned} \tag{18}$$

Where $kt_{on}/kt_{off}/kd_{off}$ stands for the binding and unbinding rate of ATP/ADP, k_{hydro} represents hydrolysis rate, k_{bin}/k_{unbin} is the binding/unbinding rate of RUVBLs to CLOCK/BMAL/CRYs. And as is described in the previous model, Nf is ratio of nuclear to cytoplasmic compartment volume.

And in this model, to close up the feedback loop, the new super-complex will replace the previous CLOCK/BMAL/CRYs complex to inhibit the transcription of E-box genes, which will be included in the $F[\lambda(t)]$ in our math model.

2.2. Phase shift effects of cordycepin on E-box gene transcription

Cordycepin is a small molecule that targets RUVBLs after phosphorylation (CoTP), shifts circadian phase in cell lines, and can significantly promote phase shift of mouse locomotor activity under jet-lag light conditions. Cordycepin is a derivative of the nucleoside adenosine, differing from the latter by the replacement of the hydroxy group in the 3' position with a hydrogen. We measured the physical interactions between RUVBL2 proteins and

CoTP/ATP by Biacore SPR assay. k_{on} of CoTP is approximately two times of ATP, and k_{off} of CoTP is approximately 1/3 to 1/4 of ATP. And since k_{on} of ADP (or CoDP) is not considered in this model, the administration of cordycepin would lead to the change in three parameters: k_{on}/k_{off} of the substrate (kt_{on} and kt_{off}), and k_{off} of the hydrolysis product (kd_{off}).

Additionally, cordycepin inhibits transcription elongation and RNA synthesis due to the absence of hydroxyl moiety at the 3' position [29], and therefore, the transcription rate of all genes will be decreased on the administration of cordycepin. The degradation of CoTP is fast, so administration of cordycepin to the whole system is regarded as a rapid inhibition of hydrolysis product dissociation from RUVBLs, and a rapid inhibition of transcription.

The effect of CoTP can be described as follow (Figure 2(a)):

$$f_i(t) = 1 + \begin{cases} 0, & t < t_0 \\ A_i \cdot (t - t_0) \cdot e^{-(t-t_0)}, & t \geq t_0 \end{cases} \tag{19}$$

Where t_0 indicates the time when cordycepin is administrated, A_i is a constant indicating the maximum fold change of variable i that is influenced by cordycepin. The pulse-like $f_i(t)$ curve mimics the dynamics of CoTP.

K_{on}/K_{off} of RUVBLs substrate (kt_{on}/kt_{off} in this model) influences two variables directly, one is the *apo* RUVBLs hexamer, another one is the hexamers bounded with ATP/CoTP. With CoTP directly repressing the dissociation of hydrolysis product, kt_{on} should experience a rapid increase, while kt_{off} decreases, and thus in this model, kt_{on} is multiplied with $f_{ton}(t)$ and kt_{off} is divided by $f_{toff}(t)$. These two variables can be described as:

$$\begin{aligned} \frac{d[\mathrm{RUVBL}^{apo}_{hex}]}{dt} =& k_{agg} \cdot [\mathrm{RUVBL}_{mono}]^6 - k_{dis} \cdot [\mathrm{RUVBL}^{apo}_{hex}], \\ & - kt_{on} \cdot f_{ton}(t) \cdot [\mathrm{RUVBL}^{apo}_{hex}] + \frac{kt_{off}}{f_{toff}(t)} \cdot [\mathrm{RUVBL}^{\mathrm{ATP}}_{hex}], \\ & + k_{unbin} \cdot [\mathrm{super-complex}] \end{aligned} \tag{20}$$

$$\begin{aligned} \frac{d[\mathrm{RUVBL}^{\mathrm{ATP}}_{hex}]}{dt} =& kt_{on} \cdot f_{ton}(t) \cdot [\mathrm{RUVBL}^{apo}_{hex}] - \frac{kt_{off}}{f_{toff}(t)} \cdot [\mathrm{RUVBL}^{\mathrm{ATP}}_{hex}], \\ & - k_{hydro} \cdot [\mathrm{RUVBL}^{\mathrm{ATP}}_{hex}] \end{aligned} \tag{21}$$

K_{off} of hydrolysis product (kd_{off} in this model) influences two variables directly, one is hexamers bounded with ADP/CoDP, the other one is

transient state of free RUVBLs hexamer. With CoTP directly repressing the dissociation of hydrolysis product, kdoff should experience a rapid decrease, and in the math model, kd_{off} is divided by $f(t)$. These two variables can be described as:

$$\frac{d[\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}]}{dt} = k_{hydro} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ATP}}] - \frac{kd_{off}}{f_{doff}(t)} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}] \tag{22}$$

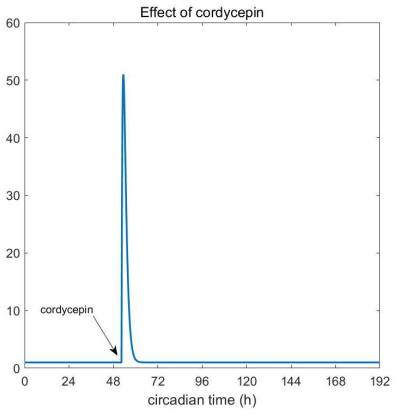
$$\begin{aligned} \frac{d[\mathrm{RUVBL}_{hex}^{apo,E}]}{dt} =& \frac{kd_{off}}{f_{doff}(t)} \cdot [\mathrm{RUVBL}_{hex}^{\mathrm{ADP}}] - k_{dis} \cdot [\mathrm{RUVBL}_{hex}^{apo,E}], \\ & - k_{bin} \cdot Nf \cdot [\mathrm{RUVBL}_{hex}^{apo,E}] \cdot [\mathrm{CLOCK/BMAL/CRYs}] \end{aligned} \tag{23}$$

And for each nucleic mRNA concentration, the transcription rate should be divided by $f_{tr}(t)$.

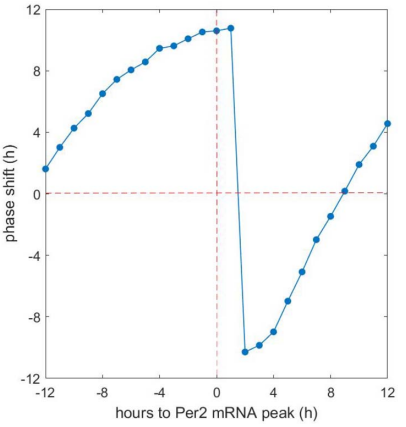
$$\frac{d[\mathrm{mRNA}_{nuc}]}{dt} = tr \cdot \frac{F[\lambda(t)]}{f_{tr}(t)} - k_f \cdot [\mathrm{mRNA}_{nuc}] - k_d \cdot [\mathrm{mRNA}_{nuc}] \tag{24}$$

According to experimental data, the timing of cordycepin treatment is important for phase shift effect, the largest effect can be achieved by adding cordycepin near the peak of $Per2-Luc$ signal, while at trough, there is little effect [8]. We tested different t_0 in our model. In this simulation, the phase shift effect is the most significant 1-2 hours after the peak of $Per2$ E-box activation, and administration of cordycepin at exactly the peak can shift phase by 10 h, which fits the experiment perfectly (Figure 2(b), Figure 2(c)).

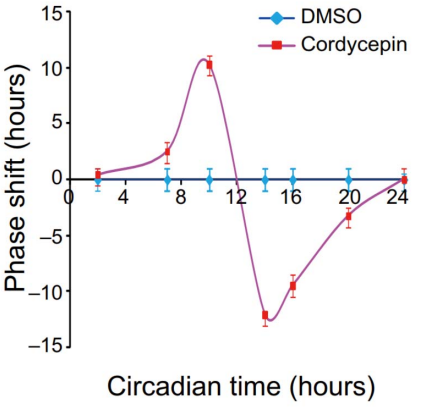
Additionally, on the administration of cordycepin, due to its transcription inhibition effects, the mRNA level of E-box genes (i.e., $Per2$, Figure 2(d)) and RORE genes (i.e., $Bmal1$, Figure 2(e)) rapidly decreases, which is also consistent with our luciferase recording data (Figure 2(f)). And on the other hand, for the inhibition of hydrolysis product dissociation from RUVBLs, the formation of high-energy transient state ($RUVBL_{apo,E}^{hex}$) is obstructed and thus inhibits the formation of super-complex (Figure 2(g)). Consequently, due to the decrease of inhibitory super complex, the activation of $Per2$ E-box transcription increases, which finally results in a delayed increase in E-box gene mRNA level (Figure 2(d)). For this super-complex only has effects on E-box motifs but not RORE, the RORE genes do not have the delayed mRNA increase. These simulation results in mRNA level are consistent with the qPCR results (Figure 2(h)).



(a) Effect of cordycepin



(b) Phase-responsive curve following cordycepin treatment by math model

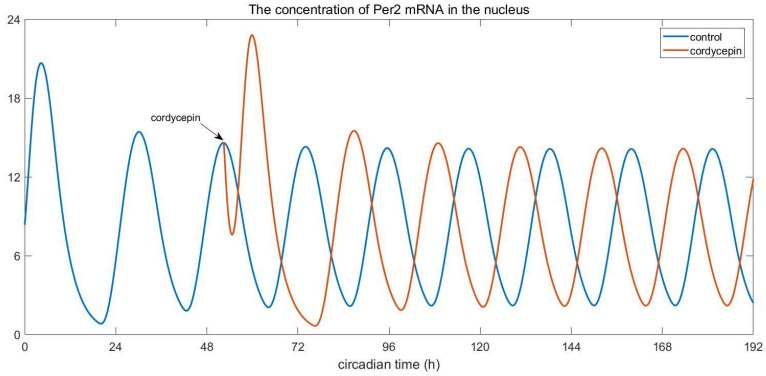


(c) Phase-responsive curve following cordycepin treatment by experiment[8]

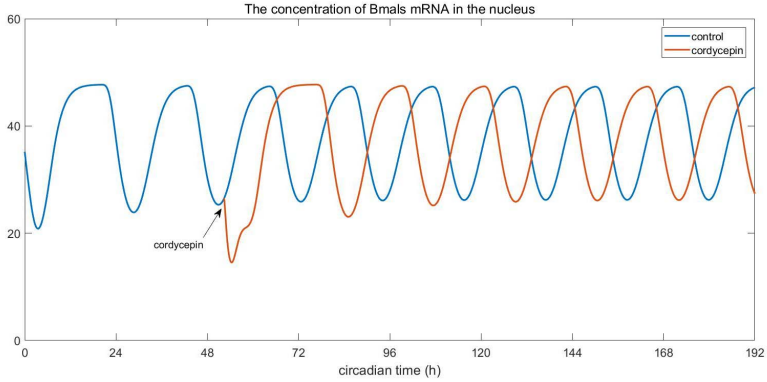
Figure 2: Time-dependent effect of cordycepin.

2.3. Sensitivity of rhythmicity to degradation rate and ATPase activity of RUVBLs

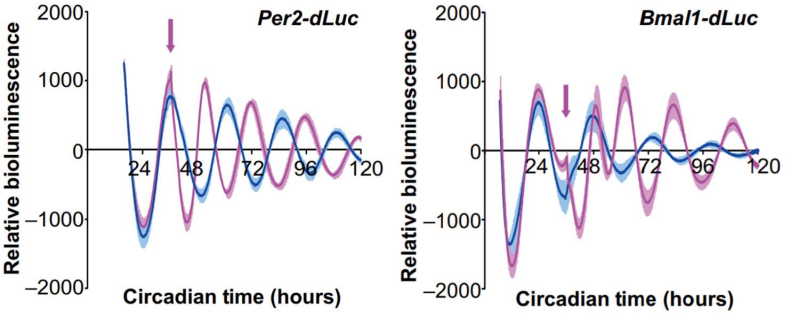
Among the parameters in the primary model, the degradation rate of proteins is the most flexible due to different protein phosphorylation states, fluctuating between 0.01 and 4. Moreover, parameters about ATP and ADP still remain unknown; therefore, we screened these parameters in a biolog-



(d) The concentration of $Per2$ mRNA in the nucleus with or without cordycepin by math model

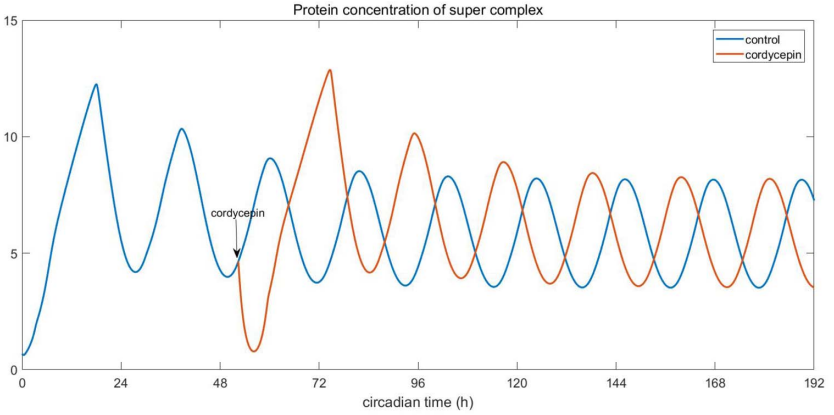


(e) The concentration of $Bmal1$ mRNA in the nucleus with or without cordycepin by math model

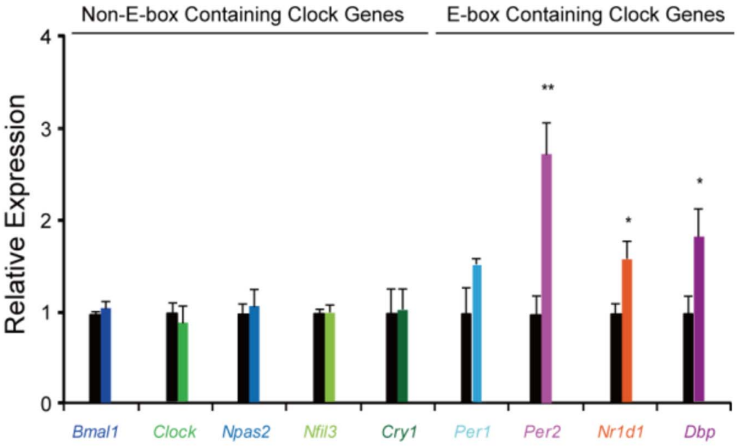


(f) The $Per2 - Luc$ and $Bmal1 - Luc$ signal with or without cordycepin by luciferase recording[8]

Figure 2: Time-dependent effect of cordycepin.



(g) Protein concentration of super-complex



(h) Relative expression of clock genes after administration of cordycepin[8]

Figure 2: Time-dependent effect of cordycepin.

ical reasonable range. From the screen, we found that degradation rate of RUVBLs monomer and the rate of ATP hydrolysis (which is indicated by hydrolysis rate, k_{hydro}) are crucial to the rhythmicity and robustness of this model, and some certain relationships exist between these two parameters and period. If we plot the heatmap (Figure 3(a)), the following information is retrieved:

- When the degradation is relatively fast, at the most suitable khydro

point (khydro=0.32, bold in Figure 3(b)), the oscillation starts to be normal, with the decrease of khydro, the amplitude will dampen and the period will be longer. With the increase of k_{hydro}, the oscillation will remain and the period will not change much (Figure 3(b)).

- When degradation is relatively low which means the monomer is steady, the period and rhythmicity do not change much with the increase of hydrolysis rate (Figure 3(c)). As the degradation rate was not measured, we cannot determine the condition that is more similar to the real condition, however, differences in period and robustness with the variation in protein stability and hydrolysis rate lead to a new question, which is role of ATP concentration and ATPase activity in circadian rhythm regulation. This question will be discussed in the discussion section.

3. Discussion

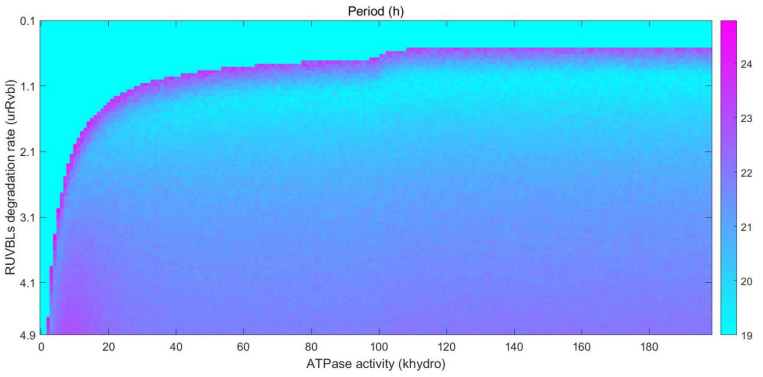
3.1. Predictions in this model and experimental proofs in previous study

3.1.1. State of RUVBLs during formation of a super-complex with other circadian components Since the participation of RUVBLs in circadian system has not been widely studied, and there are several aggregation states of the monomer, state of RUVBLs when forming super-complex with other circadian components needs to be discussed carefully.

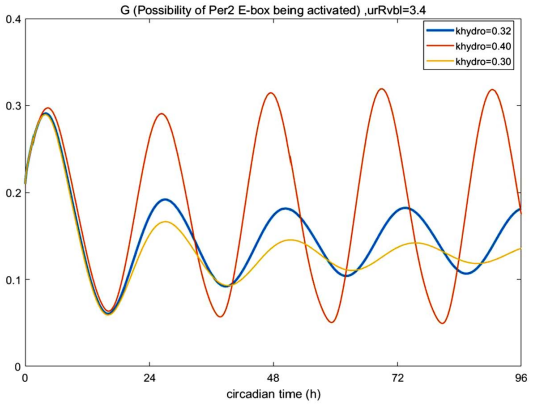
RUVBLs can be purified as monomer, pentamer, hexamer, and heptamer *in vitro*, and except for the most common hexamer, none of these aggregation states have been proved to have ATPase activity [11]. RUVBL1 and RUVBL2 can form homo-hexamer or hetero-hexamer, however, ATPase activity is the highest in RUVBL1-RUVBL2-spaced-hetero-hexamer (0.142±0.009 pmol Pi · min-1 · pmol RUVBL-1), while the other two states have a lower activity (0.015±0.001 for RUVBL1 homo-hexamer and 0.119±0.016 for RUVBL2 homo-hexamer) [12]. Furthermore, in our previous study, the RUVBLs crystal structure with CoTP, CoDP, ATP, and ADP binding is the hetero-hexamer, consequently, in this model, we chose the hexamer state to hydrolyze ATP.

The known process of ATP hydrolysis by RUVBLs hexamer is that:

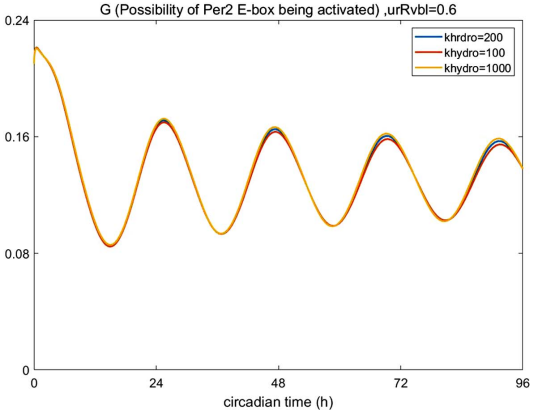
- monomers aggregate to form a ring-like hexamer;
- ATP molecules bind to the pocket on the interface of RUVBL1 and RUVBL2;



(a) Period under different RUVBLs degradation rate and ATPase activity



(b) Period variation under high RUVBLs degradation rate with different hydrolysis rate



(c) Period variation under low RUVBLs degradation rate with different hydrolysis rate

Figure 3: Sensitivity of rhythmicity to degradation rate and ATPase activity of RUVBLs.

- ATP is hydrolyzed to ADP by some negatively charged residues in the pocket;
- ADP dissociates from RUVBLs. However, it is unknown that in which step can RUVBLs bind with BMAL1 and form the super-complex.

When ATP is replaced by CoTP, RUVBLs cannot form the super-complex with BMAL1, and the RUVBL2 D299Q mutant with little ATPase activity has the same effect. The D299Q mutant blocked ATP hydrolysis, indicating that interaction with BMAL1 needs ATP hydrolysis. And since the K_d of ATP is 20 folds of CoTP [8], binding of CoTP is not possible to be blocked; there are CoDP molecules in binding pocket in crystal structure, so hydrolysis is not likely to be inhibited as well. Considering all points above, the most possible way for CoTP to disrupt the function of RUVBLs is to reduce the dissociation after hydrolysis, and consequently, the most possible state for RUVBLs to form super-complex is the apo hexamer after hydrolysis.

3.1.2. Mechanisms of phase shift effects of cordycepin In our previous published study, we used a primary detailed model without RUVBLs. The same piecewise function was used to describe the effects of cordycepin [8]. This effect function is directly multiplied by the transcription rate of E-box genes, which means that cordycepin must be a transcription enhancer to shift phase by 12 h, and in the previous model, transcriptional inhibition here cannot induce a phase shift in the same parameter range [8]. However, that model could not explain the decrease in $Per2 - Luc$ signal in our luciferase recording, and did not include the real target of cordycepin. Here, we carefully discuss multiple effects of cordycepin on circadian system and the output signals ($Per2 - Luc$ and $Bmal1 - Luc$) in this new model.

In our assumed working model of RUVBLs, on the administration of cordycepin, the most direct effect is that the release of hydrolysis product of RUVBLs is blocked. As a result, the high-energy transient state cannot form and so does the super-complex with inhibitory functions. Consequently, the activation of E-box motif should be increased at this time point, which is also true in our math model. However, activation of E-box does not equal to increasing in RNA level, for there is another step in producing RNA: RNA elongation. Free cordycepin can also act as an RNA elongation inhibitor by participating in RNA synthesis, as a result, the RNA concentration is actually decreased at the time point of cordycepin administration, in spite of the activated E-box. In luciferase recording in Figure 2(f), the $Per2 - Luc$ or $Bmal1 - Luc$ signal actually reflects the protein level of luciferase driven

by *Per*2 or *Bmal*1 promoter, and for the RNA elongation is inhibited, the protein level should also decrease immediately with cordycepin administration.

After the sudden decrease in RNA concentration, free cordycepin is soon degraded, and the RNA elongation inhibition also disappears, however, the RUVBLs are still bounded with CoDP, so the activation of E-box still exists. Consequently, *Per*2 (E-box genes) mRNA level is increased dramatically after a rapid decrease, which is consistent to the qPCR results in Figure 2(h). And since the super-complex does not bind to RORE, the mRNA level of RORE genes such as *Bmal*1 will not rebound higher than its normal peak (Figure 2(d)2(e)).

3.2. Role of ATP concentration and ATPase activity in circadian rhythm regulation

On recognizing the participation of RUVBLs into circadian regulation, we cannot ignore the fact that ATP and ATPase play an important role in circadian feedback loops. Actually, ATP concentration and ATPase activity were found to determine rhythmicity in cyanobacteria Kai system long time ago [13], however, this ATPase based circadian system has never been established in mammals, and this is the first time that we realized that ATP could be important for an organism to maintain circadian rhythm. Therefore, it is essential to study how ATP regulates circadian system, although it is hard to uncover the answer through wet experiment, math model can provide some possible clues.

In our previous study, we noticed that the D299Q mutant with little ATPase activity can induce a long-period phenotype in cells. Please note that, in Figure 3, when degradation rate of RUVBLs monomer is relatively high, period of the whole circadian system is shortened when hydrolysis activity is enhanced, and the period will be convergent to about 20 h. And with the decrease of ATPase activity, the period is lengthened, which is consistent with experiment data, and meanwhile, the amplitude is dampened. If hydrolysis activity is low enough, the system will be arrhythmic. In this case, ATPase activity is crucial factor that can control rhythmicity.

However, when the degradation rate is relatively low, the hydrolysis rate has to be in a certain range, which means both extremely high or low activity will disrupt circadian rhythm. And as the degradation becomes lower, increase in ATPase activity will not affect period anymore, indicating ATPase is not the crucial factor for rhythmicity under this condition. Note

that in this model, ATP concentration is assumed to be constant and abundant, consequently, the extremely high ATPase activity will not result in lack of ATP in this system, only in this way can period and robustness of the circadian system is able to maintain stablility. Nevertheless, in a certain organism, ATP is not abundant, and there will be a fluctuation and balance in ATP concentration or ATP/ADP ratio, which could be the decisive factor of rhythmicity.

3.3. Possibility for RUVBLs to act as a post-translational oscillator in mammalian circadian system

On discovering the participation of RUVBLs in circadian regulation, we are considering the possibility for this ATPase to be the "mammalian KaiC". Here, we discuss the similarities and differences between RUVBLs and KaiC, and the plan for our future work to provide more experimental evidence for our hypothesis.

Both RUVBLs and KaiC are slow ATPase [12, 14, 15], and due to the binding pocket of the two proteins is at the interface of two monomers and the residue with catalytic activity is on the different monomer, ATPase activity only exists in hexamers [16]. However, KaiC also has kinase activity, which can self-phosphorylate and self-dephosphorylate, and kinase activity has not been detected in RUVBLs till now. The phosphorylation state of KaiC influences the conformation of KaiC hexamer, and affect binding with KaiA and Kai B at the same time. ATP hydrolysis rate of KaiC CII domain with kinase activity is about ten folds slower than CI domain with ATPase activity, as a result, CII is the limiting step of the whole feedback loop. In the KaiC model published by Joris Paijmans, David K. Lubensky, and Pieter Rein ten Wolde in 2017 [7, 17], the difference between CI and CII is discussed, and they came up with the hypothesis that CI is the driver which is faster than CII and can provide energy, and CII is the timer which limits the period to about 24 h with the lower hydrolysis activity. Similarly, Kondo et al. also noticed the differences in hydrolysis activity in KaiC, and assumed CI and CII to be the chemical oscillator and physical oscillator.

Considering in heterozygous RUVBL1/RUVBL2 hexamer, RUVBL1 and RUVBL2 also have different ATPase activity, although they do not have kinase activity and they cannot self-phosphorylate, there might be some other proteins that can receive the hydrolyzed phosphate. Binding of ATP can influence their conformation [18], and thus influence their binding to other proteins, which is also consistent with KaiC [19].

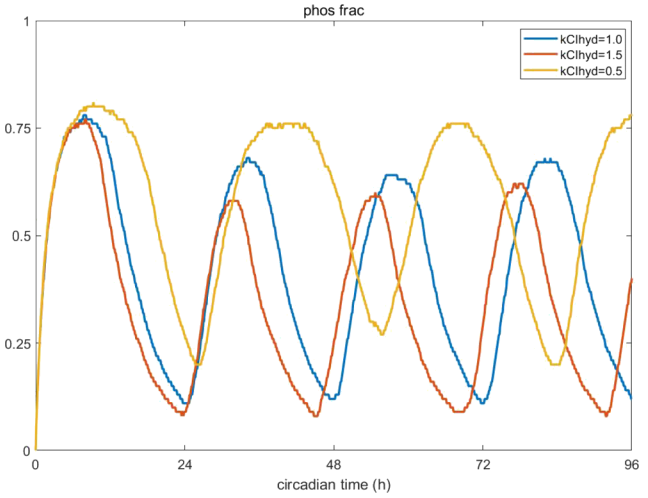


Figure 4: Period variation with different hydrolysis rate.

In the Kai math model, we also changed the hydrolysis activity, and with the increase of hydrolysis rate, the period decreased (Figure 4), which is consistent with experiment data and similar to RUVBLs system.

The origin of slowness of ATPase in KaiC and RUVBLs also needs to pay attention to. In previous studies, Jun Abe et al. noticed that the position of water molecules at the catalytic center of KaiC differs from other fast ATPase, and the far distance between water and γ-P in ATP is one of the reasons of its slowness [14]. We also conducted molecular dynamics modeling and observed the binding pocket and catalytic center of KaiC CI/CII and RUVBL1/RUVBL2, interestingly, we found that the position of Mg^{2+} in RUVBL1 is similar to CII, where Mg^{2+} is opposite to the γ-phosphate, and γ-P, Mg^{2+}, and another negatively charged residue are nearly in a line. Additionally, position of Mg^{2+} in RUVBL2 and CI are similar, where the Mg^{2+} is perfectly coordinated by the β- and γ-phosphate, and stabilizes the two phosphate and surrounding water molecules and residues, which is more beneficial for the hydrolysis [20, 21] (Figure 5). Considering the measured hydrolysis rate of KaiC CII and RUVBL1 is about 1/10 of CI and RUVBL2, we came up with a hypothesis that RUVBL1 might act as a circadian timer that gates the 24 h period like KaiC CII, and RUVBL2 might play the role of KaiC CI to provide the driving force of the circadian system.

Although we can find number of similarities between KaiC and RUVBLs, but these were based on assumptions. So, in the future, we will confirm these

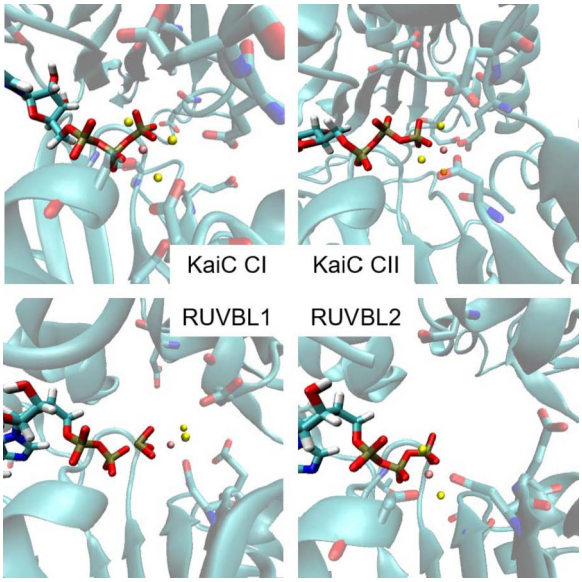


Figure 5: Position of Mg^{2+} in catalytic center of KaiC and RUVBLs.

hypotheses in the current model with biochemical and structural biological approaches.

4. Method

4.1. Modification and simulation of detailed mammal model, new variables and parameters

This model is based on the detailed model published by Jae Kyoung Kim and Daniel B Forger in 2012 [9]. Modifications are listed below:

- RUVBLs related loop is added to the detailed model including 13 new parameters and 9 new variables. New variables include the concentration of *Ruvbls* mRNA in the nucleus/cytoplasm, the concentration of RUVBLs monomer, the concentration of RUVBLs apo hexamer before/after ATP hydrolysis, the concentration of RUVBLs hexamer with ATP, the concentration of RUVBLs hexamer with ADP, and the concentration of BMAL1/CLOCK/CRYs/RUVBLs complex. New parameters include the transcription/translation rate constant, aggregation/depolymerization rate of RUVBLs monomer/hexamer, k_{on}/k_{off} of ADT/ATP, and hydrolysis rate of ATP. For the model including

Table 1: New variables

Name	Symbol
The concentration of Ruvbls mRNA in the nucleus	Mnrvbl
The concentration of Ruvbls mRNA in the cytoplasm	Mcrvbl
The concentration of RUVBLs monomer	mRvbl
The concentration of RUVBLs hexamer before ATP hydrolysis	hRvblf1
The concentration of RUVBLs hexamer with ATP	hRvblT
The concentration of RUVBLs hexamer with ADP	hRvblD
The concentration of RUVBLs hexamer after ATP hydrolysis	hRvblf2
The concentration of super-complex (cry1)	x010111
The concentration of super-complex (cry2)	x020111

Table 2: New parameters

Name	Symbol	Value
Transcription rate constant for *Ruvbls*	trRvbl	35.53
Translation rate constant for RUVBLs	tlRvbl	2.34
Binding rate constant for RUVBLs to CLOCK/BMAL/CRYs	binrvbl	15
Unbinding rate constant for RUVBLs to CLOCK/BMAL/CRYs	unbinrvbl	0.7
Degradation rate constant for Ruvbls mRNA	umRvbl	0.5
Degradation rate constant for RUVBLs protein	urRvbl	3.4
Aggregation rate constant for RUVBLs monomer	k126	0.32
Dissociation rate constant for RUVBLs hexamer	k621	0.11
Binding rate constant for ATP to RUVBLs hexamer	kton	200
Unbinding rate constant for ATP to RUVBLs hexamer	ktoff	3
Hydrolysis rate constant for the substrates of RUVBLs	khydro	0.32
Binding rate constant for ADP to RUVBLs hexamer	kdon	80
Unbinding rate constant for ADP to RUVBLs hexamer	kdoff	2
Normalized binding rate constant for BMAL-CLOCK/NPAS2 to $Cry2$ E-box	binc	1.28
Normalized unbinding rate constant for BMAL-CLOCK/NPAS2 to $Cry2$ E-box	unbinc	0.19

cordycepin, another 2 new parameters and 2 new variables are added. For detailed and graphic description, see Results and Tables (Table 1, Table 2).

- Parameter binc and unbinc (normalized binding/unbinding rate constant for BMAL-CLOCK/NPAS2 to $Cry2$ E-box) are modified to generate a better oscillation. In the primary detailed model, authors [9] differed $Cry2$ E-box from that of $Per1$, $Per2$, and $Cry1$, and the normalized binding/unbinding rate constant of $Cry2$ E-box is about 25

times lower than $Per1$, $Per2$, and $Cry1$, which is not supported by experimental data [32, 31]. So, we narrowed the gap of two types of E-box motifs.

4.2. Modification and simulation of cyanobacteria Kai model

We directly used the model published by Joris Paijmans, David K. Lubensky, and Pieter Rein ten Wolde in 2017 [7] for Kai system modeling. To test the relationship between ATPase activity and period, we only changed parameter $kCIhyd$.

4.3. Parameter estimation and screening

We have 13 new parameters in this model. Since we don't have any of the experimental data, all parameters were estimated according to other parameters in this model, and then screened in a relatively small range.

- Degradation rate constant of mRNA and monomer protein and ATP hydrolysis rate were fixed to similar to other genes.
- Aggregation/depolymerization rate of RUVBLs hexamer and K_{on}/K_{off} of ATP/ADP are set to different values and permutated and combined.
- We analyzed the rhythmicity of all generated models, and found that this model is not sensitive to aggregation/depolymerization rate of RUVBLs or kon of ADP.
- In small range screening of parameters, we found that this model is sensitive to degradation rate of protein and ATP hydrolysis rate, so we further screened these two parameters and analyzed their rhythmicity.
- Final value of parameters are chosen to make the oscillation robust, with a 23.5 h period on average.

4.4. Analysis of rhythmicity

We used a simple and quick way to distinguish the rhythmic or arrhythmic variables. For each variable in the model, we defined $x(t[i])$ as the value of this variable at time $t[i]$, where i is the number of time point in the ordinary differential equation output file. If $x(t[i]) - x(t[i-1]) > 0$ and $x(t[i+1]) - x(t[i]) < 0$, then $x(t[i])$ will be recognized as the peak value. If a certain variable has more than 4 peaks, it will be defined as rhythmic, and the time range between two peaks is calculated to be period. If all variables are rhythmic, then the parameter used in this model will be picked out. Of course, this method to calculate period is not suitable for those variables that are double-peaked within one cycle.

4.5. Molecular dynamics simulation

Molecular dynamics simulation was performed with GROMACS [22]. The initial coordinates of KaiC CI/CII and RUVBL1/2 were taken from PDB 7S66 [23] and PDB 2XSZ [16] respectively. The AMBER99SB-ILDN force field [24] was used for the proteins, and the parameters of ATP were generated by acpype [25, 26] first, and Mg^{2+} was added to the molecule according to its initial coordinates in PDB files, and the RESP charge was then calculated with Multiwfn [27]. The protein-MG-ATP complex was placed in a cubic box with periodic boundary, which was filled with TIP3P water molecules and was neutralized with 150 mM NaCl solution. The system was energy minimized until the maximum force on any atom was less than 100 $kJ/mol^{-1}nm^{-1}$, following equilibrated for 100 ps. A 50 ns molecular dynamics (MD) simulation was then completed, and RMSD was calculated. Protein-MG-ATP complex was set in one temperature controlling group, and the environment set in another group. After the 50 ns MD, the RMSD of protein is convergent.

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