A NEW REGULATORY INTERACTION SUGGESTED BY SIMULATIONS FOR CIRCADIAN GENETIC CONTROL MECHANISM IN MAMMALS

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Knowledge on molecular biological systems is increasing at an amazing pace. It is becoming harder to intuitively evaluate the significance of each interaction between molecules of the complex biological systems. Hence we need to develop an efficient mathematical method to explore the biological mechanism. In this paper, we employed hybrid functional Petri net to analyze the circadian genetic control mechanism, which is feedback loops of clock genes and generates endogenous near 24 hour rhythms in mammals. Based on the available biological data, we constructed a model and, by using Genomic Object Net, we performed computer simulations for time courses of clock gene transcription and translation. Although the original model successfully reproduced most of the circadian genetic control mechanism, two discrepancies remained despite wide selection of the parameters. We found that addition of an hypothetical path into the original model successfully simulated time courses and phase relations among clock genes. This also demonstrates usefulness of hybrid functional Petri net approach to biological systems.

1. Introduction

Virtually all physiology and behaviors in mammals show circadian rhythms; rhythms endogenously generated with period of about 24 hours. These circadian rhythms are centrally regulated by the suprachiasmatic nucleus (SCN) of the hypothalamus. Most neurons in the SCN become active during the day and are said to contain the biological clock.

With the recent discovery of the clock genes involved in the circadian rhythm, basic mechanisms for the biological clock has partially been uncovered. Several

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mathematical models have provided insight into the mechanisms of the oscillation in the negative feedback loop of the molecular circadian clock.[1,2] However, as the biological research reveals more and more complicated interactions, significance of a particular interaction among clock genes become harder to grasp.

Recently, Matsuno *et al.*[3] defined the notion of hybrid functional Petri net (HFPN) which allows us to model biological mechanism without any skills in mathematical descriptions and programming techniques. Since the HFPN has a graphical representation, our intuitive understanding of a biological mechanism can be reflected in the HFPN to be modeled, even if the biological mechanism constitutes a large network.

Genomic Object Net (GON)[4,5] is a biosimulation tool which employs the HFPN as a basic architecture. Since GON equips a user-friendly GUI system, we can easily describe an HFPN of the biological system, and manipulate the HFPN model with changing parameters to simulate the biological mechanism under various conditions. Many biological systems including λ phage genetic switch control,[6] apoptosis induced by Fas ligand,[3] *lac* operon genetic switch control,[7] and fission yeast cell cycle[8] have been modeled with GON.

This paper demonstrates how a computational model is used to understand the molecular interaction mechanism with a concrete example of the mammalian circadian clock. We constructed the HFPN model of a circadian genetic control system in mammal. Through simulations by GON on the constructed HFPN model, we evaluated the mammalian circadian genetic control system, finding two inconsistencies in oscillations of mRNAs with the known biological facts. In order to resolve these two inconsistencies, we compared the circadian genetic control systems of mammals and fruit flies. Then, we found one path of molecular interaction which exists in the circadian mechanism of fruit flies but not in that of mammals. Simulations by GON with introducing this path to the constructed HFPN model showed mRNA concentration behaviors consistent with biological observations.

2. Mammalian Circadian Genetic Control Mechanism on Biological Facts

Molecular clocks reside within SCN cells. Each molecular circadian clock is a negative feedback loop of the gene transcription and its translation into protein. The loop includes several genes and their protein products. In case of mammals, three *Period* genes (*Per1*, *Per2* and *Per3*) and two *Cryptochrome* genes (*Cry1* and *Cry2*) comprise the negative limb, while *Clock* and *Bmal1* (*Bmal*) genes constitute positive limb of the feedback loop in the molecular circadian clock.

In the morning, transcription of *Period* and *Cryptochrome* genes start to increase with the concomitant binding of CLOCK/BMAL dimers onto the E-box enhancer regions of the genes. In the afternoon, when the number of mRNA of *Period* or *Cryptochrome* genes increases, protein products (PER and CRY) encoded by the genes are actively synthesized in the cytoplasm. Although the proteins are actively

degraded by the phosphorylation, they start to move into the nucleus after the amount of the protein exceeds the threshold to form the dimers. When they enter into the nucleus, presumably in the early night, they somehow block the association of CLOCK/BMAL heterodimer or reduce their binding on the E-box sequences, thus resulting in the decrease in the transcription of *Period* and *Cryptochrome* genes. Hence in the late night, transcription of the clock genes, *Period* and *Cryptochrome* genes decrease and their protein products also decrease. Reduction of PER and CRY protein release the inhibition onto CLOCK/BMAL dimers and transcription of *Period* and *Cryptochrome* start to increase again in the next day.

This negative feedback loop of transcription and translation of clock genes is further regulated by supplementary interactions. CLOCK/BMAL dimers also induce transcription of *Rev-Erbα* (*Rev-Erb*), through the E-box sequence in the enhancer region and protein products of *Rev-Erb*, in turn, suppresses the transcription of *Bmal*.

3. Evaluation of the Present Circadian Gene Regulatory Model by Simulations

3.1. *Molecular interactions in a mathematical model*

Since biological research has been finding complicating interactions within and outside the molecular circadian mechanism, it is harder to distinguish interactions indispensable to the maintenance of oscillation from those accessory. Therefore, main purpose of this paper is to assess the significance of each interaction involved in the circadian negative feedback loop. In order to simplify the model and gain the insight of each interaction path, we deal with two group of genes (*Per1*, *Per2*, and *Per3* genes) and (*Cry1* and *Cry2* genes) collectively as *Per* and *Cry*, respectively. This is justified by similar biological effects found in knockout mouse experiments.[9]

In the present model, *Per* and *Cry* genes and their protein products constitute the first major circadian feedback loop. Second loop is composed by the *Clock* and *Bmal* genes and their protein products. These two loops are connected by the interaction including *Rev-Erb* and its product. Expression of *Rev-Erb* was accerelated by the PER/CRY dimmers and REV-ERB protein suppresses transcription of *Bmal* gene, as detailed in Figure 1.

3.2. *HFPN model of mammalian circadian gene regulatory mechanism*

Petri net[10] is a network consisting of *place*, *transition*, *arc*, and *token*. A place can hold tokens as its content. A transition has arcs coming from places and arcs going out from the transition to some places. A transition with these arcs defines a firing rule in terms of the contents of the places where the arcs are attached.

Hybrid functional Petri net (HFPN) was defined by Matsuno *et al.*[3] as an extension of hybrid Petri net (HPN).[11] HFPN allows us to model biological reactions

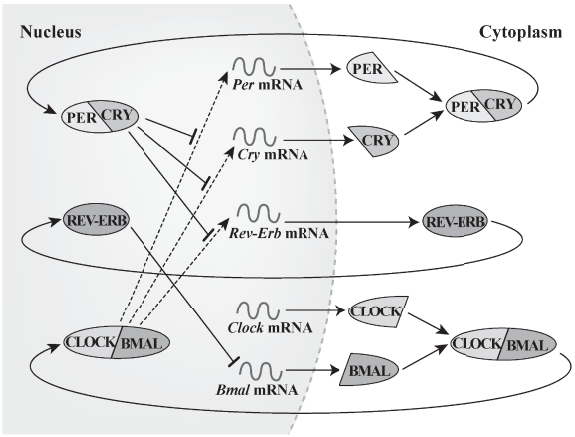


Figure 1. Interaction map of the mammalian circadian gene regulatory system. *Per* and *Cry* genes are transcribed by CLOCK/BMAL complex, translated into protein and form heterodimers before returning into cytoplasm. Products of *Clock* and *Bmal* genes bind together to play a role of the positive transcription factor for *Per*, *Cry* and *Rev-Erb* genes and their effects are counteracted by PER/CRY complex. REV-ERB protein represses transcription of *Bmal* gene.

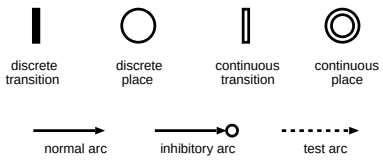


Figure 2. Elements of HFPN (HPN)

naturally than HPN. Figure 3 is an HFPN model of circadian gene regulatory mechanism in Figure 1.

Elements of HFPN (HPN) are shown in Figure 2. HFPN (HPN) has two kinds of places discrete place and continuous place and two kinds of transitions, discrete transition and continuous transition. A discrete place and a discrete transition are the same notions the traditional discrete Petri net. A continuous place can hold a nonnegative real number as its content. A continuous transition fires continuously at the speed of a parameter assigned at the continuous transition. Note that the HFPN model of this paper only uses continuous places and continuous transitions. Please refer to the literature[3,6,7] for HFPN models including discrete places and discrete transitions.

Three types of arcs are used with these places and transitions. A specific value is assigned to each arc as a weight. When a normal arc (a solid arc in Figure 3 such as the arc going into the continuous place CLOCK) with weight w is attached to a discrete/continuous transition, w tokens are transferred through the normal arc, in

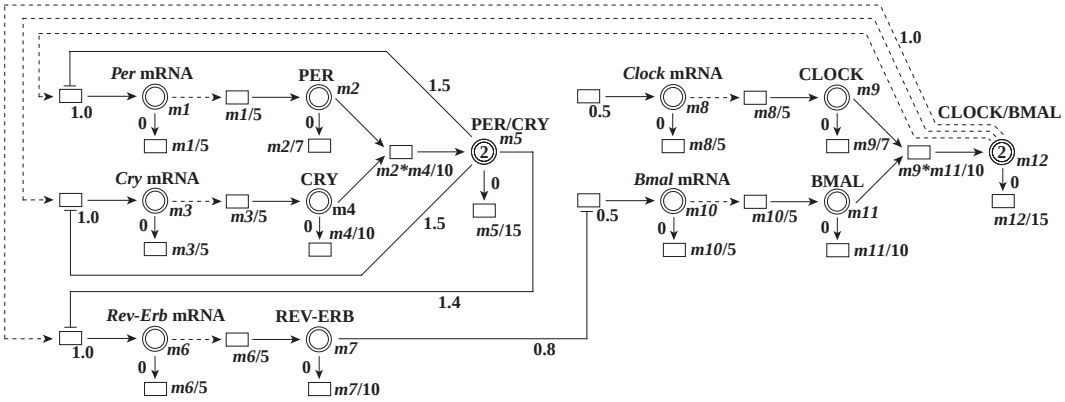
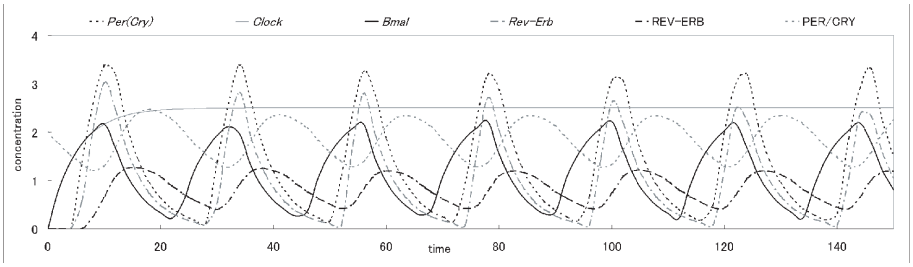


Figure 3. HFPN model of mammalian circadian gene regulatory system. Each continuous place holds the concentration of a gene product (mRNA or protein). For the continuous places with initial values greater than zero, these values are described inside the places. At the continuous transition, a speed of corresponding biological reaction is assigned. A continuous place and a continuous transition is connected by the arc which is chosen from normal arc, test arc, or inhibitory arc based on the biological interaction of target molecules. The weight of arc with no label is 0.5.

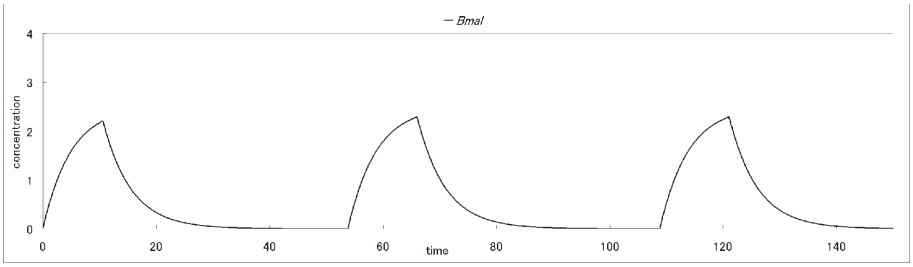
either of normal arcs coming from places or going out to places. An inhibitory arc (a line terminated with the small bar in Figure 3 such as the line coming from the continuous place REV-ERB) with weight w enables the transition to fire only if the content of the place at the source of the arc is less than or equal to w. For example, an inhibitory arc can be used to represent repressive activity in gene regulation. A test arc (a dashed line in Figure 3 such as the arc going out from the continuous place *Bmal* mRNA) does not consume any content of the place at the source of the arc by firing. For example, a test arc can be used to represent enzyme activity, since the enzyme itself is not consumed.

Figure 3 is an HFPN model being described according to the following simple rules. For each substance such as mRNA and protein, a continuous place is corresponded. At each transition, a function of the style such as $mX/10$ is assigned, which defines the speed of the corresponding reaction. For example, the translation speed of PER protein is determined by the formula $m1/5$, where $m1$ is the concentration of *Per* mRNA. This reflects the biological observation that the reaction speed of transcription is changed depending on the concentration of *Per* mRNA. Complex forming rate is given as a formula of the style such as $mX*mY/10$. For example, the formula $m2*m4/10$ is assigned at the continuous transition as the complex forming rate of the proteins PER ($m2$) and CRY ($m4$). Continuous transitions without outgoing arcs are used for representing natural degradation rate of mRNAs, proteins, and protein complexes.

After describing an HFPN of the biological mechanism to be modeled, parameters of transition speeds and initial values of places have to be determined based on the biological knowledge and/or the facts described in biological literature. In



(a) Expressions of *Bmal*, *Cry*, *Per*, *Rev-Erb*, and *Clock*



(b) Expression of *Bmal* gene when *Cry* gene is disrupted

Figure 4. Simulation results of the HFPN model in which all known biological facts are reflected. dark solid line:*Bmal* mRNA, dark dotted line:*Cry* and *Per* mRNAs, pale dot-dash-line:*Rev-Erb* mRNA, pale solid line:*Clock* mRNA, pale dotted line:PER/CRY complex, and dark dot-dash-line:REV-ERB protein. (a) *Bmal* mRNA behaves as *Per* mRNA and *Cry* mRNA, although the peak of *Bmal* mRNA is supposed to be located almost at the middle of two peaks of *Per* or *Cry* mRNA. (b) *Bmal* mRNA expresses even when *Cry* gene is disrupted.

general, many trial and error processes are required until appropriate parameters for simulation are determined. Since GON provides the GUI specially designed for biological modeling, we can perform these processes very easily and smoothly.

3.3. *Simulation results and their inconsistencies with the biological facts*

We carried out simulations of the HFPN model in Figure 4 by Genomic Object Net.[3,5] This model produces periodic oscillations of mRNA and protein concentrations as shown in Figure 4 (a). We made some modifications on this HFPN model for checking mutant behaviors including disruptions of *Per* gene (remove the normal arc going into the place *Per* mRNA) and *Cry* gene (remove the normal arc going into the place *Cry* mRNA). The resulting behavior of these modifications corresponded well to the facts in the biological literature.[15,16] However, at the same time, we found the following two inconsistencies with the biological observations.

(a) In Figure 4 (a), the *Bmal* mRNA peaks at the almost same time as the peaks of *Cry* and *Per* mRNAs. However, it is biologically known that the peak of *Bmal* mRNA is located in the almost center of two peaks of *Cry* or

Per mRNA.

(b) Figure 4 (b) shows periodical oscillation of *Bmal* mRNA in *Cry* knockout mouse. However, it contradicts the biological fact that *Bmal* gene stops oscillating in Cry knockout mouse.[9]

In order to explain the inconsistency (a), we show the following two facts and one assumption.

Fact 1 *Cry* mRNA, *Per* mRNA, and *Rev-Erb* mRNA behave similarly. That is, the differences in concentration among these three peaks are small.[12]
Fact 2 Since REV-ERB proteins repress the *Bmal* transcription, this transcription has to be stopped at the some point while the concentration of REV-ERB protein rises. In other words, the peak of *Bmal* mRNA is marked during the increase of REV-ERB protein concentration.
Assumption 1 It is known that, in the liver, the translation occurs after around 1 or 2 hours.[13] From this fact, we assume that the translation of REV-ERB protein also takes place in the SCN after around 1 or 2 hours after the *Rev-Erb* transcription.

From *Fact 1* and *Assumption 1*, we can see that the concentration of protein REV-ERB peaks 1 or 2 hours after the peaks of *Cry* or *Per* mRNA concentrations. Based on this observation and from *Fact 2*, the peak of *Bmal* concentration has to be located between the point of beginning *Cry* and *Per* transcriptions and the point in 1 or 2 hours after terminating these transcriptions.

On the other hand, the reason of the result (b) is simple. From the gene regulatory mechanism in Figure 1 it is easy to see that the *Cry* gene disruption can not contribute to block the self-feedback system of *Bmal* transcription – CLOCK/BMAL complex composition – *Rev-Erb* gene activation – repression of *Bmal* gene transcription. Thus, oscillation of the *Bmal* does not stop by the *Cry* gene disruption.

4. A New Hypothesis: PER/CRY complex activates the gene *Bmal*

Circadian clock mechanisms have been examined in many living organisms such as cyanobacteria, fruit fly, and mouse.[16,17] Especially, many investigations have been made on fruit fly (*Drosophila melanogaster*) and it is known that it has a similar circadian gene regulatory mechanism to the one in the mouse. Then, in order to fix the inconsistencies pointed out in the previous section, we compared these two circadian mechanisms. Consequently, we noticed a path in *Drosophila* circadian mechanism which has not been identified in the mouse.

- PER/TIM complex activates the gene *dClock*,[14]

where TIM (timeless) is a protein of *Drosophila* which works in place of CRY, and *dClock* is a gene of *Drosophila* which corresponds to the gene *Bmal*.

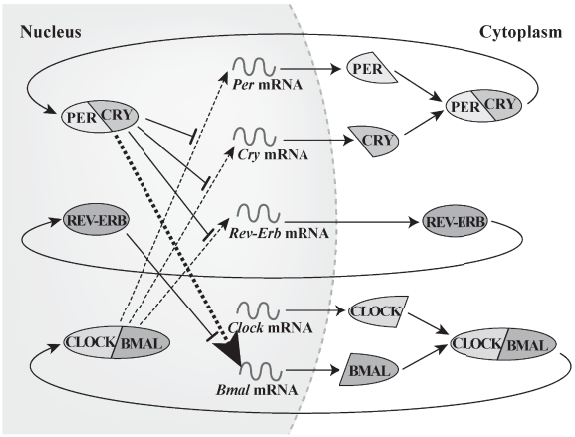


Figure 5. New Mammalian circadian gene regulatory mechanism. Inconsistencies found in the original model (Figure 1) are resolved by introducing the path of bold dotted arrow.

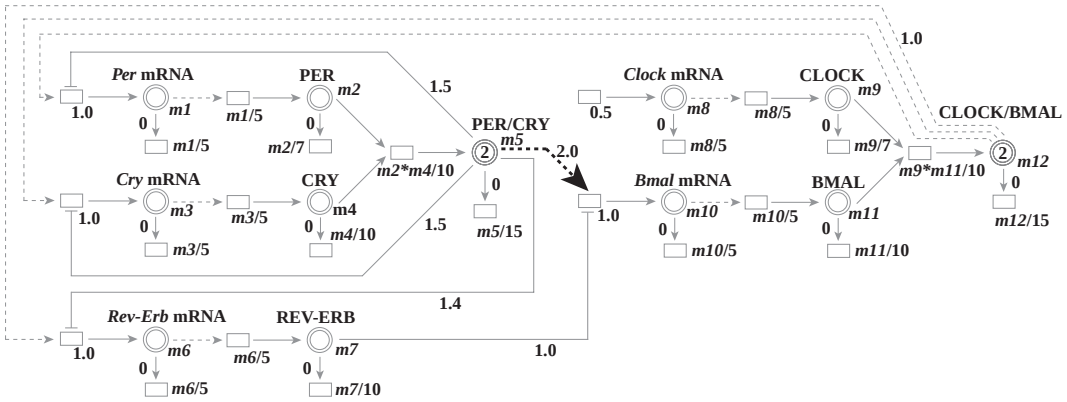
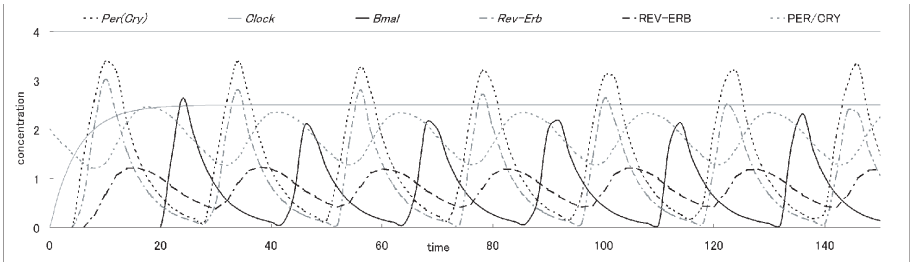


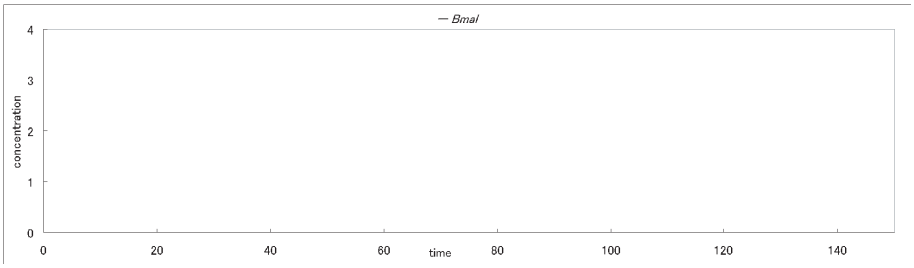
Figure 6. Improved HFPN model in which the hypothetical path (bold dotted arrow) "PER/TIM activates the gene *Bmal* is included. Refer to the caption of Figure 3 for notations.

Figure 6 is the modified HFPN model in which the above hypothetical path was incorporated by adding the bold dotted arc coming from the place PER/CRY. Figure 7 shows simulation results on this modified HFPN model. This figure shows that two biologically inconsistent points (a) and (b) presented in the subsection 3.3 have been resolved by introducing this new path.

(a) Figure 7 (a) shows the effect of the hypothetical path on the concentration behavior of *Bmal* mRNA. Recall that, in the original model, the transcription switch of gene *Bmal* was controlled only by inhibition from the REV-ERB protein. In contrast, in the new model, this transcription is controlled



(a) Expressions of *Bmal*, *Cry*, *Per*, *Rev-Erb*, and *Clock*



(b) Expression of *Bmal* gene when *Cry* gene is disrupted

Figure 7. Simulation results of the HFPN model with adding the new hypothetical reaction:PER/CRY activates the gene *Bmal*. Notation of line types is same as Figure 4. (a) The peak of *Bmal* mRNA is located almost at the middle of two peaks of *Per* or *Cry* mRNA. (b) *Bmal* mRNA does not express when *Cry* gene is disrupted.

not only the inhibition from the REV-ERB but also the activation from PER/CRY protein complex. This activation from PER/CRY complex allows the *Bmal* transcription to be off at the some point during the decrease in the PER/CRY complex concentration. In summary, the simultaneous operation of two reactions "inhibition from REV-ERB" and "activation from PER/CRY" on the gene *Bmal* enables the *Bmal* mRNA peak to locate at the middle point two *Cry* (*Per*) mRNA peaks.

(b) In order to activate *Bmal* in the new model, both of two conditions "REV-ERB represses *Bmal*" and "PER/CRY activates *Bmal*" have to be fulfilled. Thus, no oscillation of *Bmal* mRNA occurs when *Cry* is disrupted, as shown in Figure 7 (b).

5. Conclusions

Present dynamical analyses of circadian clock in mammals by HFPN model demonstrate oscillatory behaviors of clock gene expression and phase dependent phase shifts to a light pulse through temporal increase in *Per* gene transcription (in preparation). However, the original model (Figure 1) does not explain two biological established observations. First, a discrepancy found in the original model is that *Bmal* remains oscillating even in the Cry knockout mouse. Second, observation of

Bmal mRNA concentration peak could not be made at the midpoint between the peaks of *Per* or *Cry* mRNA concentration.

In order to solve these discrepancies, a new path was introduced in the original model. Introduction of the path with which PER/CRY enhance transcription of *Bmal* resolved the problems. Indeed, presence of this pathway is established in *Drosophila* and has been suggested in mammals.[15] The present finding that the positive effects of PER/CRY on transcription of *Bmal* is essential to reproduce the biologically observed behaviors of molecular circadian clock further demonstrates the usefulness of simulations on HFPN model for biological systems.

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