A MINIMAL CIRCADIAN CLOCK MODEL

ILKA M. AXMANN
i.axmann@biologie.hu-berlin.de

STEFAN LEGEWIE
s.legewie@biologie.hu-berlin.de

HANSPETER HERZEL
h.herzel@biologie.hu-berlin.de

ITB, Institute for Theoretical Biology, Humboldt University of Berlin, Invalidenstrasse 43, D-10115 Berlin, Germany

The coordination of biological activities into daily cycles provides an important advantage for the fitness of diverse organisms. Thereby, an internal circadian oscillator drives gene expression in an approximate 24 hours rhythm. Circadian clocks are found in most eukaryotes. In prokaryotes only cyanobacteria are known to regulate their activities in a circadian rhythm. *In vitro* experiments showed that three cyanobacterial proteins KaiA, KaiB and KaiC together with ATP are sufficient to generate temperature-compensated circadian oscillations of KaiC protein phosphorylation. Thus, in contrast to eukaryotic clock models the cyanobacterial core oscillator operates independently of transcription and translation processes. Most previous models of the bacterial circadian clock used complex mathematical descriptions. Here, we suggest a minimal and manageable heuristic system. Even though only four reaction steps were assumed, our model exhibited sustained oscillations of KaiC phosphorylation. A simulation of known experimental data was successful as well as oscillations maintained even for a concerted increase of Kai protein concentration. Thus, we provided a useful minimal system of differential equations which might serve as a core module of the holistic cyanobacterial clockwork in the future.

Keywords: circadian clock; oscillation; feedback; cyanobacteria; mathematical model.

1. Introduction

Numerous biological activities follow rhythmic cycles with a circa 24 hour period. Even in the absence of environmental stimuli (light, temperature) this behavior preserves a so-called free-running rhythm. The underlying core oscillator is termed circadian clock, which is ticking in most eukaryotes. The 24 h period of the free-running rhythm is not affected by normal changes in the natural environment. For several examples it is shown that the clock can compensate for perturbations within a physiological range. On the other hand, a circadian clock is able to be entrained to an environmental cycle as light [7] or temperature pulses [21].

Circadian clocks were longtime thought to be restricted to the eukaryotic kingdom. However, the optimal temporal coordination of biological processes and adaptation to daily fluctuations play an important role for diverse organisms. Green plants and phototrophic bacteria are dependent on a 12 h light - 12 h dark rhythm be-

cause of their photosynthetic activity. An internal circadian clock has been found in these photoautotrophic organisms as well. In particular, for the cyanobacterium, *Synechococcus elongatus*, a robust circadian cycling has been observed even under constant darkness. In contrast to eukaryotic clock models the cyanobacterial clock keeps operating in the presence of transcription and translation inhibitors [30]. Thus, compared to former clock models this core oscillator operates independently of transcription and translation processes. Moreover, only three different cyanobacterial proteins (KaiA, KaiB, KaiC) together with ATP are sufficient to achieve temperature-compensated 24 h rhythms of KaiC phosphorylation *in vitro* [23].

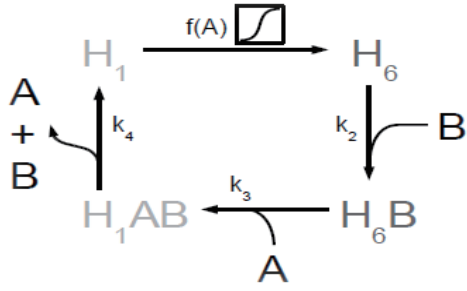

Fig. 1. Minimal model of the cyanobacterial circadian core clock comprising KaiA, KaiB and KaiC proteins. (1) Depending on the available amount of free KaiA proteins, KaiC hexamers switch between a de-(H_1) and a phosphorylated (H_6) state. (2) KaiB proteins bind to phosphorylated KaiC hexamers resulting in a conformational change of the KaiBC protein complex (H_6B). (3) KaiA proteins join the KaiBC complex. Conformational change and sequestration of free KaiA by complexation favor KaiC dephosphorylation. (4) The dephosphorylated complexes (H_1AB) break-up and release KaiA, KaiB and dephosphorylated KaiC hexamers (H_1) which restarts the circle. Default parameters of the reaction kinetics are given in Table 1.

Over the past 10 years numerous experimental investigations gave insights into molecular details of the cyanobacterial clock. Nevertheless, it remained unclear how a biochemical mechanism missing protein synthesis and degradation can keep time so precisely over long periods of several days. Now various modeling methods have been applied to the KaiABC system to simulate the chemical network that is able to generate self-sustained oscillations. In summary, multiple phosphorylation states [19], allosteric rearrangement [33], KaiA sequestration [1], monomer exchange [2, 21] or different combinations of them [29, 38] have been suggested to serve as basic mechanisms producing synchronized oscillations. Other theoretical approaches introduced a positive feedback on KaiC phosphorylation [17] or multiple states of KaiA and KaiB [15], but these models appear to be inconsistent to latest experiments [13, 21].

In order to study the recently proposed mechanism of KaiA sequestration [1] leading

to oscillation of the KaiABC clock we applied a heuristic approach. Only four reaction steps were assumed which sufficed to generate sustained oscillations. Known experimental data were simulated successfully exhibiting robust oscillatory behavior even for the concerted increase of Kai protein concentration. Thus, we defined a useful minimal model of the core circadian oscillator which might serve as a basic module to design more complex networks of the holistic cyanobacterial clockwork.

2. Model assumptions

Extensive experimental studies on cyanobacterial cells and their clock proteins revealed details about the molecular background of circadian oscillations. Here, the experimental observations were translated into a condensed mathematical model (Fig. 1). The 6 variables of the system, A, B, H_1, H_6, H_6B and H_1AB represent the concentrations of clock proteins, KaiA, KaiB, KaiC and their complexes. The dynamics of these 6 variables is described by the following system of differential equations:

$$\frac{dH_1}{dt} = -f(A) \cdot H_1 + k_4 \cdot H_1AB \tag{1}$$

$$\frac{dH_6}{dt} = f(A) \cdot H_1 - k_2 \cdot B \cdot H_6 \tag{2}$$

$$\frac{dH_6B}{dt} = k_2 \cdot B \cdot H_6 - k_3 \cdot A \cdot H_6B \tag{3}$$

$$\frac{dH_1AB}{dt} = k_3 \cdot A \cdot H_6B - k_4 \cdot H_1AB \tag{4}$$

$$\frac{dB}{dt} = k_4 \cdot H_1AB - k_2 \cdot B \cdot H_6 \tag{5}$$

$$\frac{dA}{dt} = k_4 \cdot H_1AB - k_3 \cdot A \cdot H_6B \tag{6}$$

KaiC hexamers: For our model, we considered KaiC proteins as stable hexamers (termed H) because experiments showed that this seems to represent the normal situation within the living cell [10, 20, 25]. KaiC possesses an auto-kinase and -phosphatase activity [25, 30]. Two main phosphorylation sites (T432 and S341) were described for the KaiC monomer [35], which resulted in 12 phosphorylatable sites per KaiC hexamer. **Phosphorylation:** KaiA proteins (A) were demonstrated to interact with KaiC hexamers and to enhance KaiC phosphorylation [14, 26, 31, 32, 37]. For simplification, we lumped multiple phosphorylation steps to a single reaction which is assumed to be catalyzed by KaiA. Thereby, depending on the available amount of free KaiA proteins, KaiC hexamers switch between a dephosphorylated (H_1) and a phosphorylated (H_6) state. **Conformational change:** Further it has been described that KaiB attenuates KaiA-enhanced phosphorylation of KaiC [14]. In the model, we assumed that KaiB proteins bind to highly phosphorylated KaiC hexamers. This might mediate a conformational change of the KaiBC protein complex (H_6B) so that KaiC dephosphorylation is initiated because the enhancer KaiA no longer stimulates KaiC phosphorylation. **Dephosphorylation:** As described by

different authors [6, 12], during the subjective night all three Kai proteins, KaiA, KaiB and KaiC, form stable complexes *in vivo* with yet unknown stoichiometry. In our model we assumed that KaiA joins the KaiBC complex (H_6AB) which decreases the concentration of free KaiA proteins which in turn leads to a decreased rate of KaiC phosphorylation. Thus, the sequestration of KaiA to the KaiBC complex constitutes the necessary feedback, which is indispensable for biological oscillations [1, 4]. **Break-up:** The dephosphorylated complexes (H_1AB) were assumed to become instable and to break-up. This last step led to the release of KaiA, KaiB and dephosphorylated KaiC hexamers (H_1), thus, re-starting the circle.
We chose linear and bilinear kinetics for all steps except for the KaiA-mediated KaiC phosphorylation (Fig.1). This step is assumed to depend on the concentration of free KaiA in a non-linear fashion. Our assumption seems to be justified because previous theoretical studies demonstrated [8, 9, 28] that multiple phosphorylation states result in a switch-like behavior. Thus, we modeled phosphorylation of 12 sites in the KaiC hexamer using a Hill function:

$$f(A) = \frac{f_{max} \cdot A^h}{{K_1}^h + A^h} \tag{7}$$

Using a Runge-Kutta algorithm the system of differential equations was solved numerically. MATLAB (The MathWorks, Natick, MA) implementation was helpful to analyze robustness of our model and to perform the bifurcation analysis. ASAMIN, a MATLAB gateway routine to ASA (Adaptive Simulated Annealing; www.ingber.com), was applied to fit our model to quantitative experimental data.

3. Simulations and Results

The numerical solution of our minimal model (Fig. 2) was able to generate sustained 24 hour oscillations for a chosen set of default parameters, listed in Tab. 1. The initial concentrations of Kai proteins were chosen from literature [13], which resemble the default protein concentration of the *in vitro* experiment. The simulation of the KaiC phosphorylation cycle is shown in Fig. 2 (upper graph) in the way it can be measured experimentally by time-resolved measurements of KaiC phosphorylation. The temporal development of the 6 variables of our system, A, B, H_1, H_6, H_6B and H_1AB is plotted below (Fig. 2 (lower graph)). Surprisingly, the simulated time course of KaiC phosphorylation is qualitatively very similar to experimental observations: The initial high amplitude until the system is tuned, can be found in diverse *in vitro* experiments [13, 23]. A detailed analysis of all model species (Fig. 2 (lower graph)) demonstrates that H_1 and H_6 exhibited the largest changes in concentration – dephosphorylated and phosphorylated KaiC hexamers, whereas KaiA, KaiB as well as all complexes, H_6B and H_1AB, show only small amplitude ranges (Fig. 2 (lower graph)).
Although our first simulations exhibited sustained oscillations, the sensitivity of

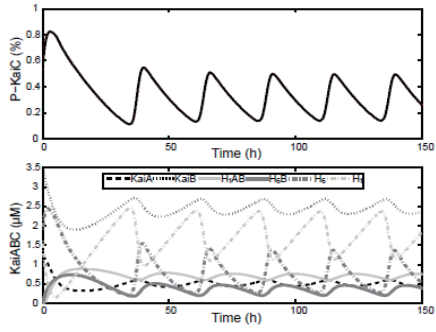

Fig. 2. The numerical solution of our minimal model using the default parameters, listed in Tab. 1. The simulation of the phosphorylated amount of KaiC (P-KaiC) in the system is shown (upper graph) exhibiting variables H_6 and H_6B normalized to the total concentration of KaiC ($KaiC_T$). This time course is qualitatively similar to *in vitro* experiments. The temporal development of the 6 variables of the system, A, B, H_1, H_6, H_6B and H_1AB is plotted below.

the system with respect to parameter changes remained to be tested. We performed a bifurcation analysis and analyzed 10-fold changes in both directions for each parameter. As main characteristics for oscillatory behavior amplitude and period were calculated (Fig. 3 left). Here, our model appeared to be robust over a 20-fold range with respect to parameters k_2 and k_4. The dephosphorylation rate, k_3, was observed to be more sensitive because oscillation were restricted to a 10-fold parameter range. We also analyzed the sensitivity of the oscillations towards the Hill coefficient in Eq. 7. These simulations clearly showed that a lower limit of $h = 11$ exists. The observed change in period was small for all parameters except for k_4,

Table 1. Model parameters

parameter	default value	interpretation
h	38	Hill coefficient
K_1	0.6 μM	ratio of phosphorylation and dephosphorylation rates
f_{max}	0.6 h^{-1}	maximal stimulatory effect
k_2	0.04 $h^{-1}\mu M^{-1}$	complex formation rate
k_3	0.4 $h^{-1}\mu M^{-1}$	dephosphorylation rate
k_4	0.1 h^{-1}	break-up rate of complexes
A_T	1.2 μM	concentration of KaiA
B_T	3.5 μM	concentration of KaiB
C_T	3.5 μM	concentration of KaiC

the break-up rate (Fig. 3 right). Here, our system exhibited the highest sensitivity to variations. This observation might indicate that complex break-up is the slowest and, therefore, the rate limiting step of our system.

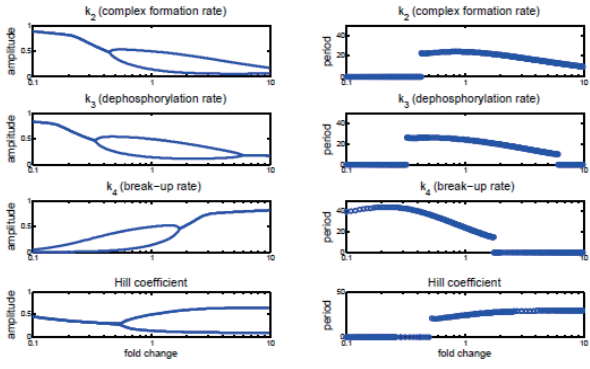

Fig. 3. Default parameters, k_2, k_3, k_4, and Hill coefficient are changed within a +/- 10-fold range from the default values given in Table 1. The observed amplitudes (left) and periods (right) are plotted visualising sensitivities and oscillation ranges.

Additionally, we tested model behavior with respect to changes of the protein concentrations. According to our bifurcation analysis shown in Fig. 4, we observed sustained oscillations for an at least 10-fold range of KaiB and KaiC concentrations as well as for a concerted variation of all three proteins. Further, it turned out that variation of KaiA concentration is the most critical element of our system. Here, amplitude and period were lost due to less than 5-fold change. The sensitivity to KaiA concentration might be explained by the fact that we assumed a KaiA-dependent Hill function for the first reaction (Eq. 7). More specifically, the concentration of free KaiA needs to cycle around the threshold parameter K_1 in order to switch phosphorylation on and off.

In summary, our results were in agreement with observations by Kageyama and colleagues [13]. They varied protein concentrations individually and showed that P-KaiC oscillations are slightly more sensitive to variation of KaiA than to KaiB which is in accordance with our simulations (compare Fig. 4 top left and 2nd row left). One still missing experiment would be the increase of KaiC protein amount for the *in vitro* experiment. Here, our model predicts oscillatory behavior even if KaiC is increased more than 10-fold, see Fig. 4 3rd row. Further, Kageyama's experiments showed that a concerted 5-fold increase in all protein concentration led to phosphorylation rhythms nearly identical to those seen under standard conditions, whereas decreased concentrations of proteins led to non-oscillatory behavior as seen

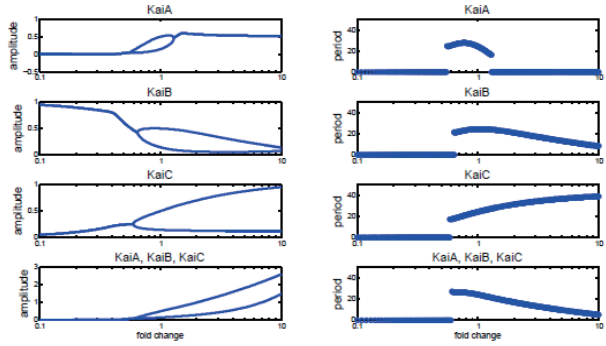

Fig. 4. Default concentrations of Kai proteins, KaiA, KaiB and KaiC, are changed 10-fold individually and for all three proteins. The corresponding amplitudes and periods are plotted visualising sensitivities and oscillation ranges.

in the model (Fig. 4 bottom left). Our model failed to reproduce Kageyama's observation that the amplitude and period remained unchanged upon a 5-fold increase of protein concentration. Using bimolecular reactions and a strong non-linearity (Eq. 7) the model appeared to be more sensitive to the variation of protein levels.

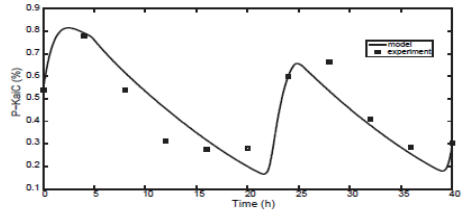

Fig. 5. Fit of our minimal circadian clock model (solid line) to quantitative experimental data (open squares) [13] using ASAMIN optimization algorithm. P-KaiC resembles the phosphorylated amount of KaiC in the system exhibiting variables H_6 and H_6B normalized to the total concentration of KaiC ($KaiC_T$). The optimized values of parameters are h = 100.0, $K_1 = 0.5838\ \mu M$, $f_{max} = 0.7033\ h^{-1}$, $k_2 = 0.0594\ h^{-1}\mu M^{-1}$, $k_3 = 0.4299\ h^{-1}\mu M^{-1}$ and $k_4 = 0.17\ h^{-1}$.

To test whether our minimal model of a circadian clock might be in quantitative agreement with experiments, we used an optimization procedure to fit the model to experimental data. Using the optimized parameter set, again our minimal model generated sustained P-KaiC oscillations. Moreover, the experimentally observed large amplitude of KaiC phosphorylation was achieved [13]; see Fig. 5.

4. Discussion and outlook

Here, we introduced a minimal model of the circadian protein oscillator of a cyanobacterial cell using a system of ordinary differential equations. The system comprised only 6 variables which represent the concentrations of clock proteins and their complexes. Thus, a system of differential equations was obtained which can be interpreted directly in biological terms because each experimentally observed protein and protein complex is given explicitly by a variable. For further simplification we focused on linear and bilinear kinetics for all steps of our model except the KaiA-mediated KaiC phosphorylation. This reaction is assumed to depend in a highly non-linear manner on the concentration of free KaiA. The assumption seems to be justified by several theoretical approaches [8, 9, 28] demonstrating that multiple phosphorylation states can result in a switch-like behavior. Thus, we included KaiA-mediated phosphorylation of KaiC by using a Hill function. Surprisingly, this minimal model was able to simulate sustained 24 hour oscillations despite the numerous simplifications. Even the large amplitude between KaiC phosphorylation and dephosphorylation level was achieved by fitting experimental data. One has to state here that a reduced model is always just a cartoon of biological reality whose predictions are expected to fail in details. For example, our cartoon of the protein clock was less successful to simulate a robust behavior of oscillation period and amplitude against concerted increase of protein concentration. When we simulated this scenario, oscillations were sustained but the period increased and the amplidude was lowered. Thus, our model appeared to be more sensitive than the real biochemical system to the variation of protein levels. Bimolecular reactions and a strong non-linearity might mediate this sensitivity to protein variations. To achieve a model as robust as the biochemical system, one or more additional steps might be included in the future. For example, an additional rate-limiting, monomolecular step (which might, e.g., represent KaiC dephosphorylation) between reactions 2 and 3 might improve the performance of our model. Much more extensive modeling approaches focusing on the cyanobacterial clock demonstrated that the robustness of circadian oscillation can be simulated. In these models, many more reaction steps were required and often unknown or less-explored states were assumed by the investigators which improved robust oscillatory behavior. To date, essential kinetic data are still not available, which makes it difficult to define a comprehensive mathematical description. Our minimal model can be extended stepwise by different kinetic mechanisms in order to get insights into the core mechanisms underlying robustness. For an oscillatory system at least one feedback and a certain delay is needed [1, 4]. In our minimal model, the sequestration of KaiA to the KaiBC complex constitutes the necessary feedback. In general, high Hill coefficients, an explicit delay or Michaelis-Menten kinetics can reduce the number of reaction steps that are needed to obtain oscillations. Our small system based on only four reactions including one non-linear step required a high Hill coefficient which kept the number of parameters in the system low. It was shown for other oscillatory systems that a necessary delay

can be caused by several processes, e.g. posttranslational modification, degradation, complex formation or nuclear import and export [27, 34]. In our case – the *in vitro* clock of Kai proteins – degradation and transport processes can be neglected. Thus, a delay might be caused by multiple phosphorylations on the KaiC hexamer modeled as a switch or by formation and break-up of Kai protein complexes. Our bifurcation analysis revealed that oscillation period was most sensitive towards the break-up rate, k_4, exhibited the highest period range. This might indicate that the break-up of complexes is the rate limiting step and, therefore, the cause of a significant delay in our system. Previous minimal models of biological oscillations such as the Goodwin model have proven to be useful tools, as they provide insights into the basic mechanism of oscillations. Also, these minimal models can easily be extended in order to understand the role of additional regulatory loops. We hope that our minimal circadian clock model in the same way might serve as a simple module to be integrated into more complex models. For example, it has been suggested that circadian timing in cyanobacteria might be additionally regulated via transcriptional / translational feedback loops [11, 16, 22, 29]. Such TTO-loops could simply be added to our core model in order to understand their impact on time keeping. The cyanobacterial Kai proteins studied here do not share sequence similarity to any known eukaryotic clock component. Nevertheless, phospho-proteins involved in circadian timing had been found in diverse organisms. Therefore, it is conceivable that a clock which is solely based on posttranslational modifications ('phoscillator' [18]) might be a general mechanism mediating circadian rhythms. Today, even for higher eukaryotes a circadian phoscillator is in discussion. Accordingly, it was shown that sustained oscillations of PER protein, a key mediator of the transcriptional feedback loop, were maintained even if its mRNA was constitutively expressed such that its coding mRNA levels were no longer under circadian control [3, 5, 24, 36]. Thus, even for eukaryotic systems transcription-translation feedback might not be the core of the circadian mechanism. Here, we suggested a useful circadian clock model which might serve as a module of holistic clockworks in the future. Compared to more complex mathematical models, in our minimal system one can comprehensively study the dynamic behavior. Our simulations confirm that KaiA sequestration can lead to self-sustained oscillations as observed experimentally.

References

[1] Clodong, S., Duhring, U., Kronk, L., Wilde, A., Axmann, I., Herzel, H., and Kollmann, M., Functioning and robustness of a bacterial circadian clock, *Mol. Syst. Biol.*, 3:90, 2007.

[2] Emberly, E., and Wingreen, N.S., Hourglass model for a protein-based circadian oscillator. *Phys. Rev. Lett.*, 96(3):038303, 2006.

[3] Fan, Y., Hida, A., Anderson, D.A., Izumo, M., and Johnson, C.H., Cycling of cryptochrome proteins is not necessary for circadian-clock function in mammalian fibroblasts, *Curr. Biol.*, 17(13):1091–1100, 2007.

[4] Friesen, W.O. and Block, G.D., What is a biological oscillator?, *Am. J. Physiol*, 246(6 Pt 2):R847–853, 1984.

[5] Fujimoto, Y., Yagita, K., and Okamura, H., Does mPER2 protein oscillate without

its coding mRNA cycling?: post-transcriptional regulation by cell clock, *Genes Cells*, 11(5):525–530, 2006.

[6] Garces, R.G., Wu, N., Gillon, W., and Pai, E.F., Anabaena circadian clock proteins KaiA and KaiB reveal a potential common binding site to their partner KaiC, *EMBO J.*, 23(8):1688–1698, 2004.

[7] Geier, F., Becker-Weimann, S., Kramer, A., and Herzel, H., Entrainment in a model of the mammalian circadian oscillator, *J. Biol. Rhythms*, 20(1):83–93, 2005.

[8] Goldbeter, A. and Koshland, D.E. Jr, An amplified sensitivity arising from covalent modification in biological systems, *Proc. Natl. Acad. Sci. USA*, 78(11):6840–6844, 1981.

[9] Gunawardena, J., Multisite protein phosphorylation makes a good threshold but can be a poor switch, *Proc. Natl. Acad. Sci. USA*, 102(41):14617–14622, 2005.

[10] Hayashi, F., Suzuki, H., Iwase, R., Uzumaki, T., Miyake, A., Shen, J.R., Imada, K., Furukawa, Y., Yonekura, K., Namba, K., and Ishiura, M., ATP-induced hexameric ring structure of the cyanobacterial circadian clock protein KaiC, *Genes Cells*, 8(3):287–296, 2003.

[11] Iwasaki, H., Nishiwaki, T., Kitayama, Y., Nakajima, M., and Kondo, T., KaiA-stimulated KaiC phosphorylation in circadian timing loops in cyanobacteria, *Proc. Natl. Acad. Sci. USA*, 99(24):15788–15793, 2002.

[12] Kageyama, H., Kondo, T., and Iwasaki, H., Circadian formation of clock protein complexes by KaiA, KaiB, KaiC, and SasA in cyanobacteria, *J. Biol. Chem.*, 278(4):2388–2395, 2003.

[13] Kageyama, H., Nishiwaki, T., Nakajima, M., Iwasaki, H., Oyama, T., and Kondo, T., Cyanobacterial Circadian Pacemaker: Kai Protein Complex Dynamics in the KaiC Phosphorylation Cycle In Vitro, *Mol. Cell*, 23(2):161–171, 2006.

[14] Kitayama, Y., Iwasaki, H., Nishiwaki, T., and Kondo, T., KaiB functions as an attenuator of KaiC phosphorylation in the cyanobacterial circadian clock system, *EMBO J.*, 22(9):2127–2134, 2003.

[15] Kurosawa, G., Aihara, K., and Iwasa, Y., A model for the circadian rhythm of cyanobacteria that maintains oscillation without gene expression, *Biophys J.*, 91(6):2015–2023, Jun 2006.

[16] Kutsuna, S., Nakahira, Y., Katayama, M., Ishiura, M., and Kondo, T., Transcriptional regulation of the circadian clock operon kaiBC by upstream regions in cyanobacteria, *Mol. Microbiol*, 57(5):1474–1484, 2005.

[17] Mehra, A., Hong, C.I., Shi, M., Loros, J.J., Dunlap, J.C., and Ruoff, P., Circadian rhythmicity by autocatalysis, *PLoS Comput. Biol.*, 2(7):e96, 2006.

[18] Merrow, M., Mazzotta, G., Chen, Z., and Roenneberg, T., The right place at the right time: regulation of daily timing by phosphorylation, *Genes Dev.*, 20(19):2629–2633, 2006.

[19] Miyoshi, F., Nakayama, Y., Kaizu, K., Iwasaki, H., and Tomita, M., A mathematical model for the Kai-protein-based chemical oscillator and clock gene expression rhythms in cyanobacteria, *J. Biol. Rhythms*, 22(1):69–80, 2007.

[20] Mori, T., Saveliev, S.V., Xu, Y., Stafford, W.F., Cox, M.M., Inman, R.B., and Johnson, C.H., Circadian clock protein KaiC forms ATP-dependent hexameric rings and binds DNA, *Proc. Natl. Acad. Sci. USA*, 99(26):17203–17208, 2002.

[21] Mori, T., Williams, D.R., Byrne, M.O., Qin, X., Egli, M., McHaourab, H.S., Stewart, P.L., and Johnson, C.H., Elucidating the Ticking of an In Vitro Circadian Clockwork. *PLoS Biol.*, 5(4):e93, 2007.

[22] Nair, U., Ditty, J.L., Min, H., and Golden, S.S., Roles for sigma factors in global circadian regulation of the cyanobacterial genome, *J. Bacteriol*, 184(13):3530–3538,

2002.

[23] Nakajima, M., Imai, K., Ito, H., Nishiwaki, T., Murayama, Y., Iwasaki, H., Oyama, T., and Kondo, T., Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro, *Science*, 308(5720):414–415, 2005.

[24] Nishii, K., Yamanaka, I., Yasuda, M., Kiyohara, Y.B., Kitayama, Y., Kondo, T., and Yagita, K., Rhythmic post-transcriptional regulation of the circadian clock protein mPER2 in mammalian cells: a real-time analysis, *Neurosci Lett.*, 401(1-2):44–48, 2006.

[25] Nishiwaki, T., Iwasaki, H., Ishiura, M., and Kondo, T., Nucleotide binding and autophosphorylation of the clock protein KaiC as a circadian timing process of cyanobacteria, *Proc. Natl. Acad. Sci. USA*, 97(1):495–499, 2000.

[26] Pattanayek, R., Williams, D.R., Pattanayek, S., Xu, Y., Mori, T., Johnson, C.H., Stewart, P.L., and Egli, M., Analysis of KaiA-KaiC protein interactions in the cyanobacterial circadian clock using hybrid structural methods, *EMBO J.*, 25(9):2017–2028, 2006.

[27] Reppert, S.M. and Weaver, D.R., Coordination of circadian timing in mammals, *Nature*, 418(6901):935–941, 2002.

[28] Salazar, C. and Hofer, T., Versatile regulation of multisite protein phosphorylation by the order of phosphate processing and protein-protein interactions, *FEBS J.*, 274(4):1046–1061, 2007.

[29] Takigawa-Imamura, H. and Mochizuki, A., Transcriptional autoregulation by phosphorylated and non-phosphorylated KaiC in cyanobacterial circadian rhythms, *J. Theor. Biol.*, 241(2):178–192, 2006.

[30] Tomita, J., Nakajima, M., Kondo, T., and Iwasaki, H., No transcription-translation feedback in circadian rhythm of KaiC phosphorylation, *Science*, 307(5707):251–254, 2005.

[31] Uzumaki, T., Fujita, M., Nakatsu, T., Hayashi, F., Shibata, H., Itoh, N., Kato, H., and Ishiura, M., Crystal structure of the C-terminal clock-oscillator domain of the cyanobacterial KaiA protein, *Nat. Struct. Mol. Biol.*, 11(7):623–631, 2004.

[32] Vakonakis, I. and LiWang, A.C., Structure of the C-terminal domain of the clock protein KaiA in complex with a KaiC-derived peptide: implications for KaiC regulation, *Proc. Natl. Acad. Sci. USA*, 101(30):10925–10930, 2004.

[33] van Zon, J.S., Lubensky, D.K., Altena, P.R., and Ten Wolde, P.R., An allosteric model of circadian KaiC phosphorylation, *Proc. Natl. Acad. Sci. USA*, 104(18):7420–7425, 2007.

[34] Vanselow, K., Vanselow, J.T., Westermark, P.O., Reischl, S., Maier, B., Korte, T., Herrmann, A., Herzel, H., Schlosser, A., and Kramer, A., Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS), *Genes Dev.*, 20(19):2660–2672, 2006.

[35] Xu, Y., Mori, T., Pattanayek, R., Pattanayek, S., Egli, M., and Johnson, C.H., Identification of key phosphorylation sites in the circadian clock protein KaiC by crystallographic and mutagenetic analyses, *Proc. Natl. Acad. Sci. USA*, 101(38):13933–13938, 2004.

[36] Yang, Z. and Sehgal, A., Role of molecular oscillations in generating behavioral rhythms in Drosophila, *Neuron*, 29(2):453–467, 2001.

[37] Ye, S., Vakonakis, I., Ioerger, T.R., LiWang, A.C., and Sacchettini, J.C., Crystal structure of circadian clock protein KaiA from Synechococcus elongatus, *J. Biol. Chem.*, 279(19):20511–20518, 2004.

[38] Yoda, M., Eguchi, K., Terada, T.P., and Sasai, M., Monomer-Shuffling and Allosteric Transition in KaiC Circadian Oscillation, *PLoS ONE*, 2:e408, 2007.