

Marshall University

Marshall Digital Scholar

Chemistry Faculty Research

Chemistry

3-28-2023


Damped Oscillating Phosphoryl Transfer Reaction in the Cyanobacterial Circadian Clock

Hye In Jang

Pyonghwa Kim

Yongick Kim

Follow this and additional works at: https://mds.marshall.edu/chemistry_faculty

 Part of the [Bacteriology Commons](#), [Biochemistry, Biophysics, and Structural Biology Commons](#), and the [Chemistry Commons](#)

Damped Oscillating Phosphoryl Transfer Reaction in the Cyanobacterial Circadian Clock

Hye-In Jang,* Pyonghwa Kim, and Yong-Ick Kim*

Cite This: *ACS Omega* 2023, 8, 10784–10788

Read Online

ACCESS |



Metrics & More

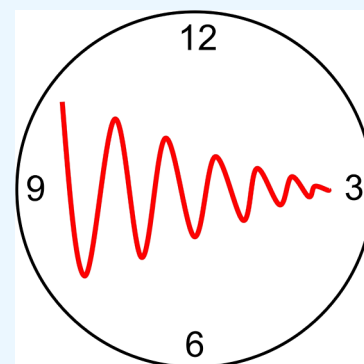


Article Recommendations



Supporting Information

ABSTRACT: Most organisms have circadian clocks to ensure the metabolic cycle to resonate with the rhythmic environmental changes without “damping,” or losing robustness. Cyanobacteria is the oldest and simplest form of life that is known to harbor this biological intricacy. Its KaiABC-based central oscillator proteins can be reconstituted inside a test tube, and the post-translational modification cycle occurs with 24 h periodicity. KaiC’s two major phosphorylation sites, Ser-431 and Thr-432, become phosphorylated and dephosphorylated by interacting with KaiA and KaiB, respectively. Here, we mutate Thr-432 into Ser to find the oscillatory phosphoryl transfer reaction damps. Previously, this mutant KaiC was reported to be arrhythmic in vivo. However, we found that the mutant KaiC gradually loses the ability to run in an autonomous manner and stays constitutively phosphorylated after 3 cycles in vitro.



INTRODUCTION

All life on earth has been subjected to the cosmic event of earth rotating around the sun. Attempts to study what this cyclic process has brought upon various organisms on this planet have revealed that all domains of life from bacteria to humans have developed an ability to anticipate these events instead of merely reacting to them. The discovery of this biochemical timekeeping has established the concept of circadian clock that controls 24 h rhythm of a host. The overarching idea of transcriptional–translational feedback loop (TTFL), which is known to be present in all timekeeping organisms, is that the abundance of clock proteins oscillates with 24 h periodicity to signal information about the time of day, controlling the gene expression pattern to resonate with the environment for ensuring better survivability.¹

This is also true for cyanobacteria, in which its metabolic activities are controlled by an accurate circadian clock, an internal time-keeping system in a cell.² However, in a cyanobacterial strain, *Synechococcus elongatus* PCC 7942, the 24 h rhythm has been found to be generated by the circadian oscillator, composed of only three major proteins—KaiA (uniProt ID: Q79PF6), KaiB (uniProt ID: Q79PF5), and KaiC (uniProt ID: Q79PF4)—which runs in a manner somewhat independent of TTFL.³ The biochemical aspects of the proteinaceous circadian clock have been studied in detail using the in vitro reconstitution method, which has revealed the chemical mechanism among the clock components.^{4–7} During the day and night, two residues of KaiC—Ser 431 (Ser⁴³¹) and Thr 432 (Thr⁴³²)—undergo phosphorylation and dephosphorylation, respectively (Figure 1). For phosphorylation, Thr⁴³² gets phosphorylated before Ser⁴³¹. In the falling

phase of the clock, Thr⁴³² gets dephosphorylated first, and Ser⁴³¹ is next.^{8,9} Meanwhile, the mechanistic details of the ordered phosphorylation of KaiC still remain questionable.

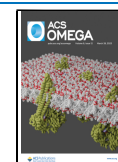
In KaiC, there is a linear chain relatively far away from the Ser⁴³¹ and Thr⁴³² called “A-loop”.¹⁰ KaiA binds KaiC and activates its phosphorylation by holding the A-loop into its exposed conformation, modulating the binding affinity of the magnesium ion (Mg²⁺) in KaiC’s active site.¹¹ This enables KaiC to initiate autophosphorylation. KaiB deactivates the phosphorylation by sequestering KaiA from A-loop and activates dephosphorylation by binding on the N-terminal domain of KaiC (CI).¹²

As long as adenosine triphosphate is supplied, the 24 h oscillatory phosphorylation of KaiC sustains for days when mixed with KaiA, KaiB, and Mg²⁺ inside a test tube.^{6,7} Thus, this cyclic post-translational modifications of KaiC are considered the central gears and cogs of timekeeping in cyanobacteria, and TTFL is known to contribute to robustness.¹³ It has long been a question whether the KaiC phosphorylation is ordered due to the amino acid-specific effect. To study the mechanistic importance of KaiC residues undergoing phosphorylation and dephosphorylation—Ser⁴³¹ and Thr⁴³²—we mutated Thr⁴³² to Ser (Ser⁴³²) and monitored its oscillating properties. Here, we report that the mutation on

Received: October 6, 2022

Accepted: March 2, 2023

Published: March 14, 2023



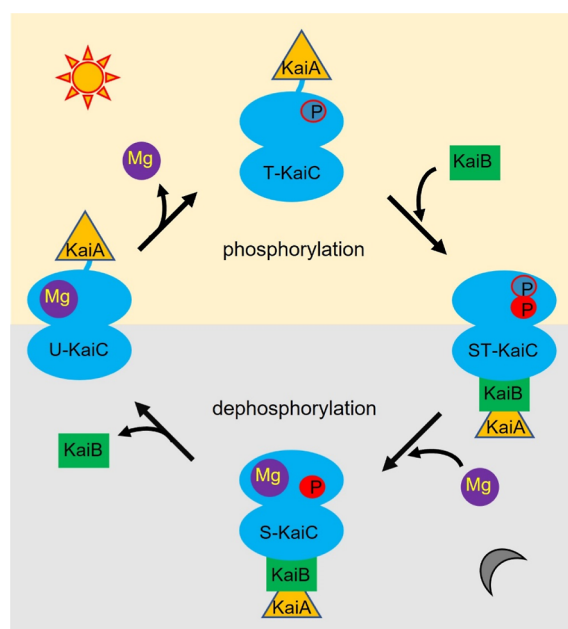


Figure 1. Oscillating phosphoryl-transfer reaction of the circadian oscillator protein KaiC. When KaiC is unphosphorylated (U-KaiC), KaiA binds to the A-loop of KaiC. After Thr gets phosphorylated (P in the red open circle), Ser becomes phosphorylated (P in the red closed circle). KaiB binds to the fully phosphorylated KaiC (ST-KaiC) and sequesters KaiA away from the A-loop. Binding magnesium ion (Mg^{2+}) to ST-KaiC activates the dephosphorylation of KaiC. Thr is dephosphorylated first (S-KaiC) and Ser is next. When KaiC is fully dephosphorylated (U-KaiC), KaiB is dissociated from KaiC and KaiA can bind to the A-loop. Dissociation of Mg^{2+} activates phosphorylation and another cycle starts.

KaiC generates a “damped” oscillation on the phosphorylation of KaiC, meaning that its oscillation is not sustained and has an amplitude that diminishes over a few cycles.

MATERIALS AND METHODS

Cloning, Expression, and Purification of KaiC. The mutation on KaiC was performed by site-directed mutagenesis. Two synthesized oligos were used as primers of the polymerase chain reaction (PCR). The sequences are 5′-CTGACTCC-CATATCTCATCAATTACGGAT ACGATTATC-3′ and 5′-GATAATCGTATCCGTAATTGATGAGATATGGGAGT-CAG-3′. The pET-41a(+) wt-KaiC plasmid previously made was used as a template.⁷ The verified sequence plasmid was transformed in *Escherichia coli* (BL21DE3, Novagen) to overexpress the protein. The culture was grown in 1 L of Luria Bertani (LB) media containing kanamycin at 37 °C. When OD₆₀₀ of the culture reached 4.0, the temperature was lowered to 25 °C for 1 h. 100 μ M IPTG was added to induce the overexpression of ss-KaiC, and the culture was incubated for additional 16 h. Cells were harvested by centrifugation at 5500g for 10 min. Cell pellets were resuspended in 60 mL of wash buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM ATP, and 1 mM DTT at pH 7.3) and lysed by passing through a chilled French press cell at a constant rate with 16,000 psi. The lysates were centrifuged at 20,000g for 2 h and filtered through a 0.45 μ m filter (Thermo Fisher). The separation of KaiC from crude cell lysate was achieved by passing through a GST column (GSTrap HP, GE Healthcare). After the sample elution with the elution buffer

(50 mM Tris–HCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 1 mM DTT, and pH 7.3), PreScission protease (GE Healthcare) was used to cleave the GST tags from KaiC. The final KaiC protein isolate was obtained using another GST column. A desalting column was used to switch to the reaction buffer (150 mM NaCl, 20 mM Tris–HCl, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, and pH 8.0).

Oscillating Phosphoryl Transfer Reaction. The oscillating reaction mixture was prepared by mixing 1.2 μ M KaiA, 3.5 KaiB, 3.5 μ M KaiC, 150 mM NaCl, 1 mM ATP, 5 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM Tris–HCl at pH 8.0.^{6,7} After aliquoting from each reaction during evenly spaced 2 h time points, the samples were denatured, run on a 6.5% SDS–polyacrylamide gel, and analyzed by constructing a densitometric graph of phosphorylation states using ImageJ (NIH).¹⁴

RESULTS

To phosphorylate or dephosphorylate ss-KaiC, the reaction mixture was incubated overnight at 30 °C in the absence of KaiA or KaiB, respectively. KaiA or KaiB was added in the treated reaction mixture to initiate the oscillating phosphoryl transfer reaction.

To generate the mutation, we applied the site-directed mutagenesis to replace Thr⁴³² to Ser⁴³² (ss-KaiC). The oscillating properties are monitored with ss-KaiC and compared with wild-type KaiC (wt-KaiC). All experimental conditions were followed by the previously published protocol.^{6,7} The two phosphorylation sites (Ser⁴³¹ and Ser⁴³²) in ss-KaiC are known to be phosphorylated in vitro.¹⁵

Non-oscillatory phosphorylation and dephosphorylation profiles of ss-KaiC were similar to those of wt-KaiC. ss-KaiC underwent dephosphorylation in the absence of KaiA and KaiB (Figures 2A and S1A). With KaiA, ss-KaiC got phosphorylated

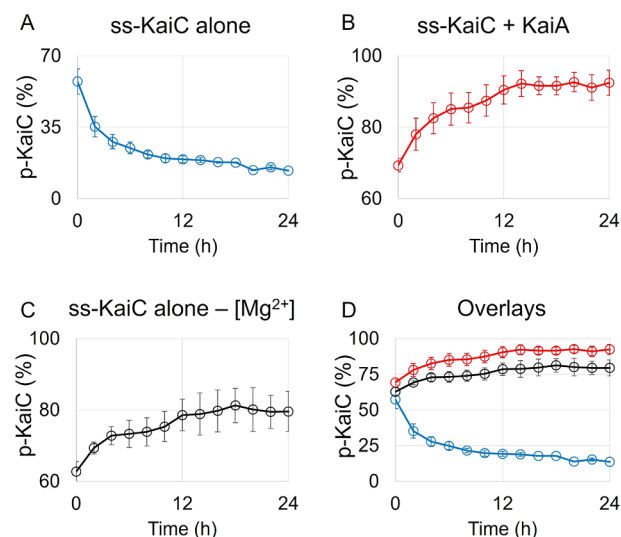


Figure 2. Phosphorylation and dephosphorylation profiles of ss-KaiC. (A) Dephosphorylation of ss-KaiC in the absence of KaiA. (B) Phosphorylation of ss-KaiC in the presence of KaiA. (C) Phosphorylation of ss-KaiC in the absence of KaiA and Mg^{2+} . (D) Overlay of (A–C). p-KaiC represents the percentage of phosphorylated KaiC. Open circles represent the average of each data point of two replicates. The vertical bars on the data points represent the standard error of the mean (SEM). Lines connecting each data point were drawn to show the progress of the reaction. The original protein gel pictures are shown in the Supporting Information.

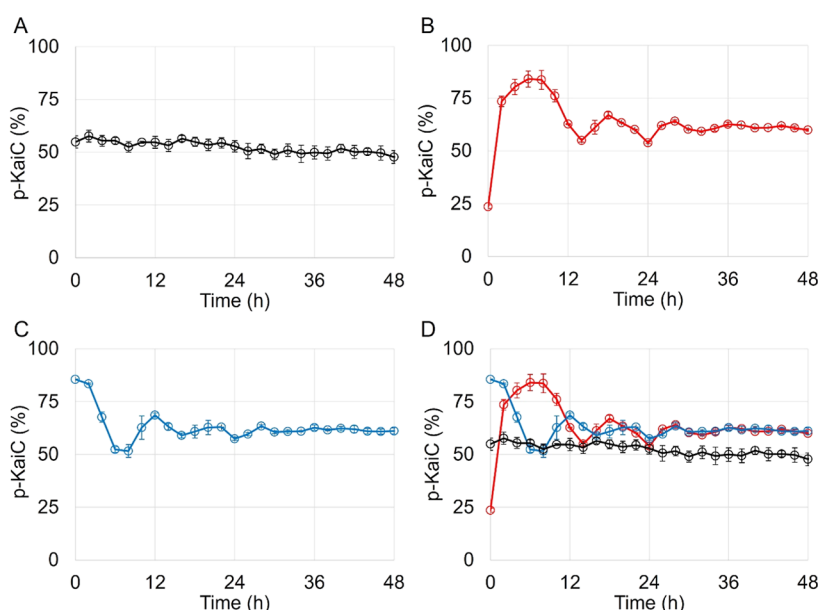


Figure 3. Oscillating phosphorylation reaction of ss-KaiC. (A) KaiA and KaiB were mixed with freshly purified ss-KaiC at time 0. (B) KaiA was mixed in the reaction mixture containing mostly dephosphorylated ss-KaiC and KaiB at time 0. (C) KaiB was mixed in the reaction mixture containing mostly phosphorylated ss-KaiC and KaiA at time 0. (D) Overlay of (A–C). All represent the same as Figure 2.

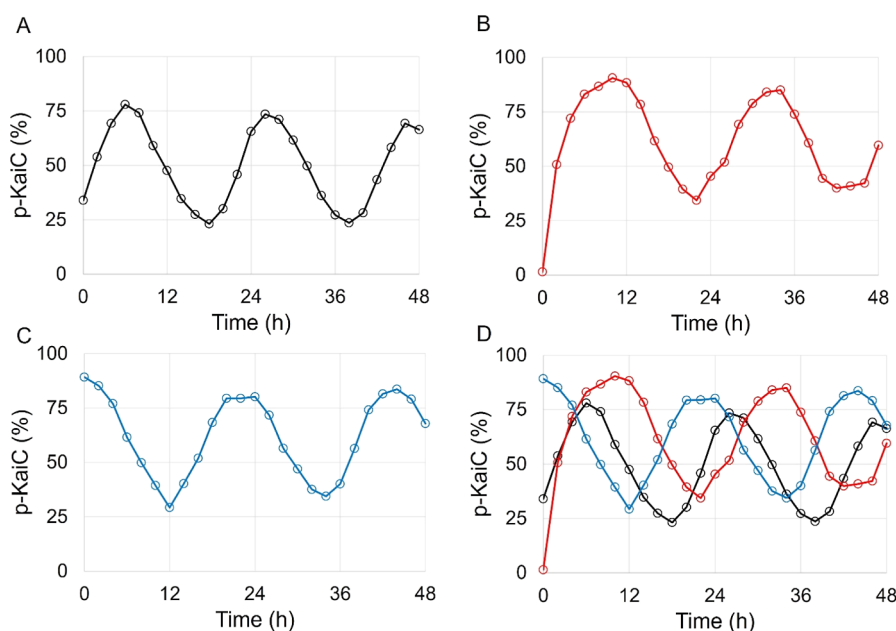


Figure 4. Oscillating phosphorylation reaction of wt-KaiC. (A–D) All are the same as Figure 3 except replacing ss-KaiC with wt-KaiC. All graphs are generated by a single experiment.

(Figures 2B and S1B). We recently reported that KaiC alone undergoes phosphorylation in the absence of Mg^{2+} in the reaction mixture.^{11,16} Accordingly, ss-KaiC underwent phosphorylation in the absence of Mg^{2+} (Figures 2C and S1C). Therefore, we concluded that there is no intrinsic mutant-specific property in ss-KaiC regarding the KaiC-alone phosphorylation and dephosphorylation pattern (Figure 2D).

Also, we tested the oscillating reaction profile of ss-KaiC by including KaiA and KaiB in the reaction mixture. The freshly purified ss-KaiC was mixed with KaiA and KaiB at time 0 to monitor the phosphorylation pattern. Surprisingly, the freshly purified ss-KaiC in the reaction mixture did not show the oscillation, in contrast to wt-KaiC (Figures 3A and S2A). We

reasoned that the freshly purified ss-KaiC did not undergo phosphorylation or dephosphorylation because it had already reached the equilibrium between the two antagonistic reactions.¹⁶ To shift the equilibrium, we dephosphorylated ss-KaiC by incubating it with KaiB at 30 °C overnight. At time 0, KaiA was added into the reaction mixture containing dephosphorylated ss-KaiC. The ss-KaiC shows the damped oscillating pattern under this reaction condition (Figures 3B and S2B). Although we were unable to fully dephosphorylate ss-KaiC (it starts with ~25% of phosphorylation), it was enough to shift the equilibrium toward the phosphorylation phase. The damped oscillation was sustained for three cycles with a shorter period (~12 h) than that of wt-KaiC (~24 h).

To check the oscillating property with different initial phosphorylation states, we tried to phosphorylate ss-KaiC before mixing the reaction mixture. We can phosphorylate ss-KaiC by incubating it with KaiA at 30 °C overnight. We added KaiB in the incubated reaction mixture at time 0 to monitor the phosphorylation state. As in the previous case, the phosphorylation state of ss-KaiC showed oscillation (Figures 3C and S2C). The oscillation properties are similar to that of dephosphorylated ss-KaiC except that they are in the opposite phase (Figure 3D).

To verify that the damped oscillation is a genuine property of ss-KaiC, we performed the same experiments with wt-KaiC. wt-KaiC shows the sustained oscillation under all different initial conditions without changes in any oscillating properties (Figures 4 and S3).

CONCLUSIONS

In conclusion, we found that the single mutation (Thr⁴³² to Ser⁴³²) on the first phosphorylated residue in KaiC generates a damped oscillation. Previously, this mutant was reported as arrhythmic in vivo.¹⁵ The discrepancy with our result may come from the different initiation due to other clock components already present within the cell. Our result also shows that the oscillation is arrhythmic until we initiate the oscillation (Figure 3A). It is possible that the two opposite enzymatic modes of KaiC already reached equilibrium before measuring the oscillation in vivo.

This mutation helps elucidate the reaction mechanism of the oscillating phosphorylation reaction in KaiC. It is also questionable whether the order of ss-KaiC phosphorylation is retained as in wild-type KaiC.⁸ Further examination of ss-KaiC may reveal the mechanism of the ordered phosphorylation. Intriguingly, excess KaiA in the reaction mixture results in a damped oscillation with a shortened period and constitutive phosphorylation state.^{17,18} However, this type of damped oscillation shows a constitutively hyper-phosphorylated state. Our damped oscillation of ss-KaiC must be a different mechanism because the oscillation maintains an overall phosphorylation level of 50–60% (Figure 3). Also, the damped oscillations are conserved in different KaiA concentrations (Figure S4). We infer that the Ser replacement results in premature phosphorylation or inefficient dephosphorylation, since ss-KaiC with KaiA and KaiB always maintained the phosphorylation state higher than 50%. Because Thr contains the methyl group, the Ser mutation only gives an effect of removing the methyl group. We argue that removing the methyl group in Thr may change the overall phosphorylation rate and causes the sustained oscillation to damp, although further characterization should be performed to propose the detailed mechanism of the time-keeping reaction.

It is also possible that the 12 h damped oscillation may have been brought by the inefficient “autonomous synchronization process”, a concept suggested by Ito et al.¹⁹ The idea behind this is that each KaiC monomer exchanges itself across hexamers to allosterically induce a homogeneous phosphorylation state of the KaiC hexamer. KaiC’s monomer exchange activity can be tested by applying the parameters to the mathematical model suggested by Sasai.²⁰ Although the mechanistic elucidation of the autonomous synchronization process was not studied, our findings on ss-KaiC may contribute to further structural and functional study of the autonomous synchronization.

One of the hallmarks of all circadian clocks is that they continue unabated in a constant environment. Interestingly, a different strain of cyanobacteria, called *Prochlorococcus marinus*, developed a less sophisticated KaiBC-based clock that merely reacts to the environmental changes.^{21,22} *Prochlorococcus* KaiC damps when exposed to constant light and stays constitutively phosphorylated without KaiA.²³ More details are needed to relate the work on *Prochlorococcus* to the damping pattern of phosphoryl transfer reaction observed in *S. elongatus* ss-KaiC. Nonetheless, this work will contribute to understanding how the circadian clock autonomously ticks even without any input from the environment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c06457>.

SDS–gel images of ss-KaiC and wt-KaiC and densitometry graphs and SDS–gel images of ss-KaiC (PDF)

Accession Codes

General information about KaiA, KaiB, and KaiC proteins can be obtained in the UniProt (www.uniprot.org) with accession codes Q79PF6, Q79PF5, and Q79PF4, respectively.

AUTHOR INFORMATION

Corresponding Authors

Hye-In Jang – School of Cosmetic Science and Beauty Biotechnology, Semyung University, Jecheon 27136, Republic of Korea; Email: inijjang@semyung.ac.kr

Yong-Ick Kim – Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, New Jersey 07102, United States; orcid.org/0000-0001-5491-616X; Email: ykim@njit.edu

Author

Pyeonghwa Kim – Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, New Jersey 07102, United States; Present Address: Laboratory of Genetics, The Rockefeller University, New York, NY, USA;

Complete contact information is available at: <https://pubs.acs.org/10.1021/acsomega.2c06457>

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

This work was supported by the Semyung University Research Grant of 2020.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Hana Nim for the insightful discussions.

ABBREVIATIONS

TTF, transcriptional–translational feedback loop; Thr, threonine; Ser, serine; IPTG, isopropyl β -D-1-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; ATP, ad-

enosine triphosphate; DTT, dithiothreitol; GST, glutathione S-transferase

REFERENCES

- (1) Dunlap, J. C. Molecular bases for circadian clocks. *Cell* **1999**, *96*, 271–290.
- (2) Kim, P.; Kaur, M.; Jang, H. I.; Kim, Y. I. The Circadian Clock-A Molecular Tool for Survival in Cyanobacteria. *Life* **2020**, *10*, 365.
- (3) Tomita, J.; Nakajima, M.; Kondo, T.; Iwasaki, H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* **2005**, *307*, 251–254.
- (4) Nakajima, M.; Imai, K.; Ito, H.; Nishiwaki, T.; Murayama, Y.; Iwasaki, H.; Oyama, T.; Kondo, T. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* **2005**, *308*, 414–415.
- (5) Kim, P.; Kim, Y.-I. An In Vitro Approach to Elucidating Clock-Modulating Metabolites. In *Circadian Rhythms in Bacteria and Microbiomes*; Johnson, C. H., Rust, M. J., Eds.; Springer International Publishing: Cham, 2021; pp 203–220.
- (6) Kim, Y.-I.; Boyd, J. S.; Espinosa, J.; Golden, S. S. Detecting KaiC Phosphorylation Rhythms of the Cyanobacterial Circadian Oscillator In Vitro and In Vivo. In *Methods in Enzymology*; Sehgal, A., Ed.; Academic Press, 2015; Vol. 551, pp 153–173.
- (7) Kim, P.; Kaszuba, A.; Jang, H. I.; Kim, Y. I. Purification of GST-Fused Cyanobacterial Central Oscillator Protein KaiC. *Appl. Biochem. Microbiol.* **2020**, *56*, 395–399.
- (8) Rust, M. J.; Markson, J. S.; Lane, W. S.; Fisher, D. S.; O’Shea, E. K. Ordered phosphorylation governs oscillation of a three-protein circadian clock. *Science* **2007**, *318*, 809–812.
- (9) Nishiwaki, T.; Satomi, Y.; Kitayama, Y.; Terauchi, K.; Kiyohara, R.; Takao, T.; Kondo, T. A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J.* **2007**, *26*, 4029–4037.
- (10) Kim, Y. I.; Dong, G.; Carruthers, C. W., Jr.; Golden, S. S.; LiWang, A. The day/night switch in KaiC, a central oscillator component of the circadian clock of cyanobacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 12825–12830.
- (11) Jeong, Y. M.; Dias, C.; Diekman, C.; Brochon, H.; Kim, P.; Kaur, M.; Kim, Y. S.; Jang, H. I.; Kim, Y. I. Magnesium Regulates the Circadian Oscillator in Cyanobacteria. *J. Biol. Rhythms* **2019**, *34*, 380–390.
- (12) Chang, Y. G.; Cohen, S. E.; Phong, C.; Myers, W. K.; Kim, Y. I.; Tseng, R.; Lin, J.; Zhang, L.; Boyd, J. S.; Lee, Y.; Kang, S.; Lee, D.; Li, S.; Britt, R. D.; Rust, M. J.; Golden, S. S.; LiWang, A. Circadian rhythms. A protein fold switch joins the circadian oscillator to clock output in cyanobacteria. *Science* **2015**, *349*, 324–328.
- (13) Teng, S. W.; Mukherji, S.; Moffitt, J. R.; de Buyl, S.; O’Shea, E. K. Robust circadian oscillations in growing cyanobacteria require transcriptional feedback. *Science* **2013**, *340*, 737–740.
- (14) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671–675.
- (15) Egly, M. Architecture and mechanism of the central gear in an ancient molecular timer. *J. R. Soc., Interface* **2017**, *14*, 20161065.
- (16) Kim, P.; Thati, N.; Peshori, S.; Jang, H.-I.; Kim, Y.-I. Shift in Conformational Equilibrium Underlies the Oscillatory Phosphoryl Transfer Reaction in the Circadian Clock. *Life* **2021**, *11*, 1058.
- (17) Nakajima, M.; Ito, H.; Kondo, T. In vitro regulation of circadian phosphorylation rhythm of cyanobacterial clock protein KaiC by KaiA and KaiB. *FEBS Lett.* **2010**, *584*, 898–902.
- (18) Kaur, M.; Ng, A.; Kim, P.; Diekman, C.; Kim, Y. I. CikA Modulates the Effect of KaiA on the Period of the Circadian Oscillation in KaiC Phosphorylation. *J. Biol. Rhythms* **2019**, *34*, 218–223.
- (19) Ito, H.; Kageyama, H.; Mutsuda, M.; Nakajima, M.; Oyama, T.; Kondo, T. Autonomous synchronization of the circadian KaiC phosphorylation rhythm. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1084–1088.
- (20) Sasai, M. Mechanism of autonomous synchronization of the circadian KaiABC rhythm. *Sci. Rep.* **2021**, *11*, 4713.
- (21) Johnson, C. H.; Zhao, C.; Xu, Y.; Mori, T. Timing the day: what makes bacterial clocks tick? *Nat. Rev. Microbiol.* **2017**, *15*, 232–242.
- (22) Holtzendorff, J.; Partensky, F.; Mella, D.; Lennon, J. F.; Hess, W. R.; Garczarek, L. Genome streamlining results in loss of robustness of the circadian clock in the marine cyanobacterium *Prochlorococcus marinus* PCC 9511. *J. Biol. Rhythms* **2008**, *23*, 187–199.
- (23) Axmann, I. M.; Dühring, U.; Seeliger, L.; Arnold, A.; Vanselow, J. T.; Kramer, A.; Wilde, A. Biochemical evidence for a timing mechanism in *prochlorococcus*. *J. Bacteriol.* **2009**, *191*, 5342–5347.

Recommended by ACS

General Method of Quantifying the Extent of Methionine Oxidation in the Prion Protein

Christopher J. Silva and Melissa L. Erickson-Beltran

JANUARY 06, 2023

JOURNAL OF THE AMERICAN SOCIETY FOR MASS SPECTROMETRY

READ 

To Explore the Binding Affinity of Human γ -Secretase Activating Protein (GSAP) Isoform 4 with APP-C99 Peptides

Deekshi Angira, Vijay Thiruvankatam, et al.

MARCH 27, 2023

ACS OMEGA

READ 

In Silico Characterization of *Withania coagulans* Bioactive Compounds as Potential Inhibitors of Hydroxymethylglutaryl (HMG-CoA) Reductase of *Mus m...*

Muhammad Bilal Azmi, Nazia Feroz, et al.

JANUARY 29, 2023

ACS OMEGA

READ 

In Silico-Based Structural Evaluation to Categorize the Pathogenicity of Mutations Identified in the RAD Class of Proteins

Aaliya Anwaar, Reshita Baruah, et al.

MARCH 08, 2023

ACS OMEGA

READ 

Get More Suggestions >