



AFRL-AFOSR-VA-TR-2016-0294

ADP Regulates the Structure and Function of the Protein KaiC

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08/11/2016
Final Report

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REPORT DOCUMENTATION PAGE			<i>Form Approved</i> OMB No. 0704-0188		
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1. REPORT DATE (DD-MM-YYYY) 11-08-2016		2. REPORT TYPE Final Performance		3. DATES COVERED (From - To) 01 Aug 2013 to 31 Jul 2017	
4. TITLE AND SUBTITLE ADP Regulates the Structure and Function of the Protein KaiC			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER FA9550-13-1-0154		
			5c. PROGRAM ELEMENT NUMBER 61102F		
6. AUTHOR(S) Andy LiWang			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF CALIFORNIA MERCED 5200 N LAKE RD MERCED, CA 95343-5001 US			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) AF Office of Scientific Research 875 N. Randolph St. Room 3112 Arlington, VA 22203			10. SPONSOR/MONITOR'S ACRONYM(S) AFRL/AFOSR RTB2		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) AFRL-AFOSR-VA-TR-2016-0294		
12. DISTRIBUTION/AVAILABILITY STATEMENT A DISTRIBUTION UNLIMITED: PB Public Release					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The LiWang lab has published two papers under this grant (Chang et al., 2015; Tseng et al., 2014), and has a third paper currently under peer review at the journal Science. In the paper by Tseng et al., undergraduate researchers are authors including one Hispanic undergraduate student. The paper by Chang et al., published in Science, received accolades: (Mori et al., 2015), www.rsc.org/chemistryworld/2015/06/molecular-machinery-behind-circadian-clocks-ticking-revealed , http://phys.org/news/2015-06-scientists-decipher-tick-tock-biological-clocks.html . Moreover, this grant has spawned several new directions in circadian-clocks research for the LiWang lab, as will be described in the Research Summary section. The discoveries of the LiWang lab are summarized in a recent YouTube video (https://youtu.be/DcuKifCRx_k). For this animation, the LiWang lab worked closely with the instructor of the undergraduate BioClock Studio course at UC San Diego (http://ccb.ucsd.edu/the-bioclock-studio/index.html). There were iterative cycles of animation by undergraduates and suggested edits by the LiWang lab to produce the published video.					
15. SUBJECT TERMS Protein					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF	19a. NAME OF RESPONSIBLE PERSON BRADSHAW, PATRICK
a. REPORT	b. ABSTRACT	c. THIS PAGE			
<small>Standard Form 298 (Rev. 8/98) Prescribed by ANSI Std. Z39.18</small>					

DISTRIBUTION A: Distribution approved for public release.

Unclassified	Unclassified	Unclassified	UU	PAGES	19b. TELEPHONE NUMBER <i>(Include area code)</i> 703-588-8492
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FINAL REPORT

This report describes three years (08/01/2013 – 07/31/2016) of accumulated work from the LiWang lab, which is supported by AFOSR grant FA9550-13-1-0154.

Introduction

Under this grant, the LiWang lab investigated the molecular mechanism of the circadian clock of cyanobacteria. They are the only organisms thus far whose clock can be reconstituted in a test tube without the complex milieu of living cells (Nakajima et al., 2005). The LiWang lab exploited this in vitro clock system to gain an atomic-resolution understanding of a circadian clock. The rationale is that fundamental principles of timekeeping are shared among diverse life forms (just like all aircraft share basic principles of flight).

The LiWang lab has published two papers under this grant (Chang et al., 2015; Tseng et al., 2014), and has a third paper currently under peer review at the journal *Science*. In the paper by Tseng et al., undergraduate researchers are authors including one Hispanic undergraduate student. The paper by Chang et al., published in *Science*, received accolades: (Mori et al., 2015), www.rsc.org/chemistryworld/2015/06/molecular-machinery-behind-circadian-clocks-ticking-revealed, <http://phys.org/news/2015-06-scientists-decipher-tick-tock-biological-clocks.html>. Moreover, this grant has spawned several new directions in circadian-clocks research for the LiWang lab, as will be described in the Research Summary section. The discoveries of the LiWang lab are summarized in a recent YouTube video (https://youtu.be/DcuKifCRx_k). For this animation, the LiWang lab worked closely with the instructor of the undergraduate BioClock Studio course at UC San Diego (<http://ccb.ucsd.edu/the-bioclock-studio/index.html>). There were iterative cycles of animation by undergraduates and suggested edits by the LiWang lab to produce the published video.

Circadian clocks consist of 1) an oscillator that generates a biochemical rhythm with a circadian period, 2) output pathways that transmit clock signals to control gene expression, 3) and resetting pathways that receive environmental cues to synchronize the oscillator to local time. The oscillator of cyanobacteria is composed of three proteins, KaiA, KaiB, and KaiC, which, when mixed together with ATP, generate a sustained circadian rhythm of interactions that can be observed using various methods (Figure 1).

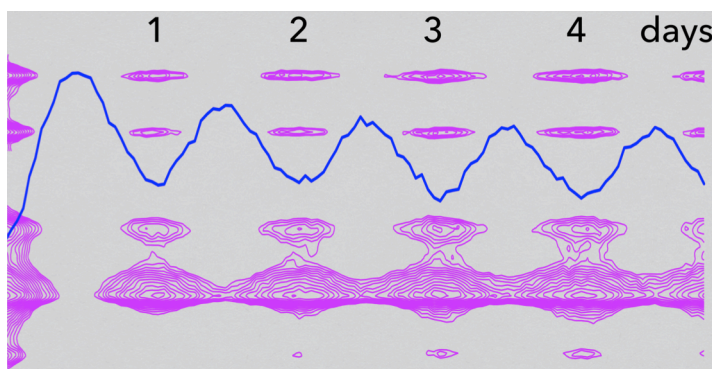


Figure 1. Real-time NMR data, collected by the LiWang lab, on a mixture of recombinant KaiA, KaiB, and KaiC proteins and ATP. This figure is a stack plot of one-dimensional spectra of the methyl region. (Unpublished.)

Output proteins SasA and CikA interact with oscillator proteins to transmit time-of-day information to the transcription factor, RpaA, to generate global rhythms in gene expression (Gutu and O’Shea, 2013). The Research Summary section will describe work by the LiWang lab on how these proteins communicate with the oscillator proteins.

At the beginning of each day, KaiA stimulates KaiC to autophosphorylate (Williams et al., 2002) (Figure 2). During this time, KaiC stimulates the clock-output protein kinase, SasA, to phosphorylate the transcription factor, RpaA, which then activates expression of clock-controlled genes (Gutu and O’Shea, 2013). KaiB resides in an inactive form referred to as the ground state, or gsKaiB (Chang et al., 2015). At dusk, KaiC reaches a hyperphosphorylated state. This state of KaiC binds to the active, fold-switched state of KaiB, or fsKaiB (Chang et al., 2015). Once bound to KaiC, KaiB prevents KaiA from stimulating KaiC by sequestering KaiA. With KaiA inactivated, KaiC starts to autodephosphorylate throughout the night. KaiB also stimulates the clock-output protein phosphatase, CikA, to inactivate RpaA. By dawn, KaiC is hypophosphorylated, whereupon it releases KaiB, which in turn releases KaiA, thereby initiating a new cycle of KaiC phosphorylation.

Thus, circadian rhythms in gene expression are generated by cycles of RpaA activation by SasA, followed by its inactivation by CikA. The big questions for the LiWang lab are how these clock proteins interact, how these interactions cause changes in their activities, and how they are able to rhythmically assemble and disassemble with precision throughout the day and night.

During the three years under AFOSR support, the LiWang group investigated how the oscillator proteins described in Figure 2 work together to generate a rhythm with a slow 24-hour period, and how they interact with output proteins to transmit timing signals. Details of their findings are presented in the Research Summary section.

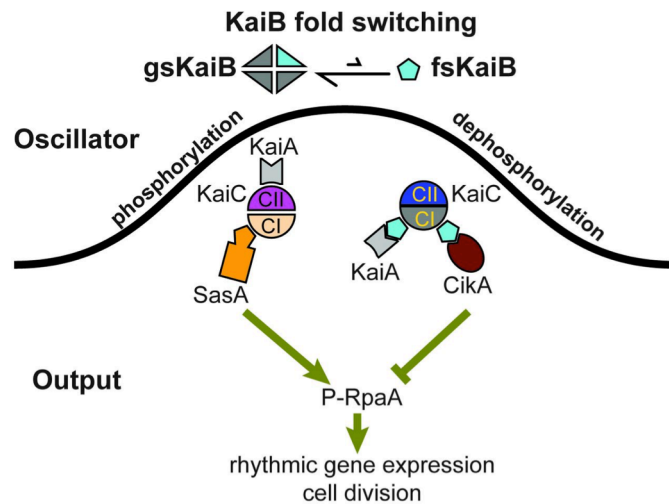


Figure 2. The circadian clock of cyanobacteria drives circadian rhythms of gene expression. During the day, KaiA stimulates KaiC to autophosphorylate, and KaiC stimulates the kinase SasA, which then activates the transcription factor RpaA. KaiB resides in the inert ground state (gsKaiB). When KaiC becomes hyperphosphorylated, it binds the fold-switched state of KaiB (fsKaiB), which then sequesters and inactivates KaiA. fsKaiB also stimulates the phosphatase CikA, which inactivates RpaA. From Tseng et al. (2014).

Broader Impacts

The LiWang lab has extended the impact of every dollar awarded to it by the AFOSR by mentoring middle school, high school, and undergraduate students, many of whom are underprivileged/disadvantaged, in science and research. Since 2009, the LiWang lab has continuously participated in the following broader-impact activities in the San Joaquin Valley of California:

- American Chemical Society's Summer Experience for the Economically Disadvantaged (SEED) Project. (<https://www.acs.org/content/acs/en/education/students/highschool/seed.html>)

Every summer, the LiWang lab mentors 1–2 high school students from the local community in hands-on research projects. They are from families at or below the poverty line, and want to attend college to pursue a STEM field. The research experience is often transformative for these young students.

- Merced County Office of Education, Dinner with a Scientist. (<http://video.valleypbs.org/video/2365483927/>)

Every spring, the Merced County Office of Education hosts a three-hour event, including dinner, where local middle school students have informal conversations with scientists. The goal of the event is to reveal to these young students how fun it is to make scientific discoveries.

- Undergraduate research in the LiWang lab at UC Merced (a Hispanic-Serving Institution: <http://www.hacu.net/assnfe/cv.asp?ID=1411>).

At any point in time, there are between 1–4 undergraduate students in the LiWang lab carrying out research projects. Because UC Merced is a Hispanic-Serving Institution, with 48.5% of the student body identifying as Hispanic, many of LiWang's undergraduate researchers are Hispanic. On a peer-reviewed research article from the LiWang lab, a Hispanic undergraduate student was an author, as were two non-Hispanic undergraduates (Tseng et al., 2014).

The LiWang lab is located in the San Joaquin Valley (SJV) of California. This culturally and ethnically diverse region trails the rest of California in most socioeconomic and environmental indicators. In 2016, a report by the New York Times showed that children in this region lag 1.5 grade levels behind the national average:

www.nytimes.com/interactive/2016/04/29/upshot/money-race-and-success-how-your-school-district-compares.html

Census data from the 1970s onward show that approximately 45% of about 4 million SJV residents have emigrated from Central and Latin America to this agricultural region (California Department of Finance, 2007). In Merced County, where UC Merced is located, 53% of the county population is Hispanic (US Census data from 2010, <http://www.census.gov/>). Merced County is challenged by 24.8% poverty (www.census.gov/quickfacts/table/PST045214/06047), as opposed to 14.8% statewide (www.census.gov/quickfacts/table/PST045215/00). Out of 58 total counties in California, Merced County has the fourth highest unemployment rate (www.labormarketinfo.edd.ca.gov/file/lfmonth/countyur-400c.pdf). Typically employed as agricultural workers or in other low-paying jobs, this population is mired in chronic poverty that becomes difficult to overcome. The SJV has some of the lowest college-attending rates of high school graduates in California, and has been determined to be the most historically underserved area in California.

Thus, STEM-related outreach activities of the LiWang lab in the SJV broadens and diversifies the impact of AFOSR funding.

Research Summary

Significant discoveries by the LiWang lab are described in chronological order in this section. Thus, it begins with published results, and finishes with as-yet unpublished work. Each discovery is summarized by a bullet point, and followed by relevant details.

2013 – 2014:

- The linker region of KaiA is essential for KaiA-KaiB binding.

The LiWang lab showed that the linker regions, and not the N-terminal domains, of KaiA (Figure 3) bind to KaiB (Tseng et al., 2014). This finding was significant because it corrected work from another lab that mistakenly suggested that KaiA uses its N-terminal domains to bind KaiB (Pattanayek et al., 2011). The LiWang lab also demonstrated that KaiA uses a distinctly different region, its C-terminal domains, to bind KaiC. (As will be described later, in 2016, the LiWang lab collaborated with an X-ray crystallographer to confirm that the linker regions of KaiA indeed bind to KaiB.)

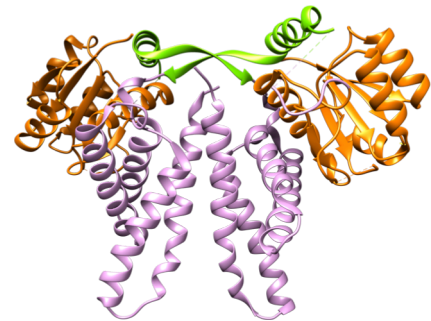


Figure 3. Crystal structure of KaiA (Ye et al., 2004). Orange: N-terminal domains; green: linker regions; purple: C-terminal domains.

- KaiA-KaiB-KaiC interactions are cooperative.

Using both NMR and fluorescence spectroscopy, the LiWang lab showed that KaiA enhances KaiB-KaiC binding, and KaiC enhances KaiA-KaiB binding (Tseng et al., 2014). This cooperative formation of the nighttime KaiA-KaiB-KaiC complex explained the robustness (i.e., switch-like behavior) of the day-to-night phase transition by the clock.

- Stoichiometry of the KaiA-KaiB complex.

The LiWang lab used NMR spectroscopy and size-exclusion chromatography to show that one KaiB monomer binds to one KaiA dimer (Tseng et al., 2014). This finding was significant because it rectified a previous report that suggested that a dimer of KaiB binds to KaiA (Mutoh et al., 2010). The stoichiometry determined by the LiWang lab could explain the known dependency of the phosphorylation rhythm on KaiA and KaiB concentrations (Nakajima et al., 2010), whereas the incorrect stoichiometry could not. (In 2016, the LiWang lab collaborated with an X-ray crystallographer to confirm that the stoichiometry they determined in Tseng et al. (2014) is indeed correct.)

- The mechanistic basis of maintaining phase coherence by the clock.

A major question in the chronobiology field was how a population of *microscopic* KaiA, KaiB, and KaiC proteins maintains a *macroscopic* circadian rhythm without losing coherence in the presence of stochastic thermal fluctuations (e.g., see Figure 1). The LiWang lab solved this mystery by finding that as KaiC progressively autophosphorylates on residue S431 under stimulation by KaiA, the KaiA-KaiC affinity weakens (Tseng et al., 2014). Therefore, KaiA proteins have a tendency to stimulate autophosphorylation of KaiC proteins that are phosphorylated to a lesser extent relative to other KaiC proteins. This discovery was significant, because it explained how KaiC proteins, under stimulation by KaiA, autophosphorylate coherently in the presence of thermal noise.

- Cooperativity of KaiA-KaiB-KaiC assembly regulates clock-output signaling.

SasA and KaiB compete for binding to similar sites on KaiC. However, the LiWang lab found that the presence of KaiA swings the competition in favor of KaiB, as a result of cooperative assembly of the KaiA-KaiB-KaiC complex (Tseng et al., 2014). This finding showed that cooperative KaiA-KaiB-KaiC assembly adds robustness (switch-like behavior) to clock-output signaling at the day-to-night transition.

- KaiB and SasA bind to the B-loops on the CI domains of KaiC.

In 2014, it was still controversial as to the location of the KaiB-binding site on KaiC. Indeed, several studies suggested that KaiB binds to the CII domain of KaiC (Pattanayek et al., 2013; Pattanayek et al., 2008; Pattanayek et al., 2011; Snijder et al., 2014; Villarreal et al., 2013). Only the LiWang lab reported that KaiB binds to the CI domain of KaiC (Chang et al., 2012). Then, in Tseng et al. (2014), the LiWang lab reaffirmed that KaiB does indeed bind to CI and not CII. In that paper, they narrowed down the binding region to an exposed loop on the CI domain they named the B-loop (Figure 4). (In 2016, the LiWang group collaborated with an X-ray crystallographer to prove that KaiB binds to the B-loop on the CI domain of KaiC.)

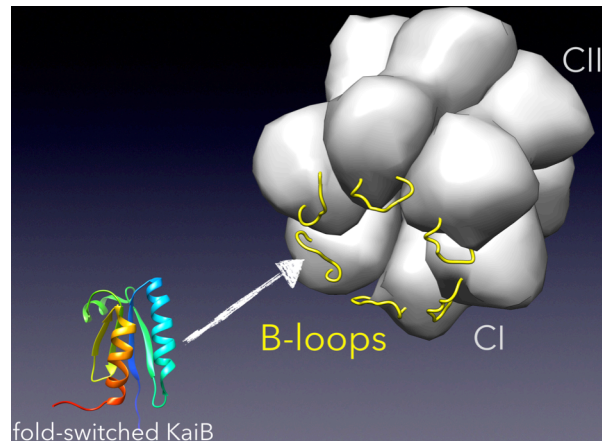


Figure 4. Illustration showing that KaiB binds to the B-loops on the CI domains of KaiC. These loops were discovered by the LiWang lab by aligning the homologous amino acyl sequences of the CII and CI domains, and noticing an insertion of several residues in CI that was missing in CII.

2014 – 2015:

- KaiB must switch from an inactive fold to an active fold to bind KaiC.

A major conundrum was the inability to explain why it takes hours for KaiB to bind KaiC, even when residue S431 of KaiC was phosphorylated, which is the signal for KaiB-KaiC binding. This problem was significant because the slowness of KaiB-KaiC binding was essential for the generation of the slow 24-hour rhythm. The many published crystal structures of KaiB are basically identical (Garces et al., 2004; Hitomi et al., 2005; Iwase et al., 2005; Pattanayek et al., 2008; Villarreal et al., 2013). These structures failed to explain the slowness of KaiB-KaiC binding, however. The breakthrough occurred when the LiWang lab solved the NMR structure of KaiB when it was bound to the CI domain of KaiC, and discovered that it had a completely different fold than that found in the crystal structures of free KaiB (Chang et al., 2015). Thus, KaiB switches its fold to bind KaiC. KaiB belongs to the rare class of so-called metamorphic proteins, which reversibly flip between two distinct folds under native conditions (Murzin, 2008). Less than 10 proteins are currently classified as metamorphic. The energy barrier between the two folds of KaiB slows down the rate of KaiB-KaiC binding. The published crystal structures of free KaiB are of the inactive form, and often referred to by the LiWang lab as the ground-state, or gsKaiB. The fold of KaiB when it is bound to KaiC is the active state and is often referred to as the fold-switched state, or fsKaiB. See Figure 5 for a side-by-side comparison of gsKaiB and fsKaiB structures.

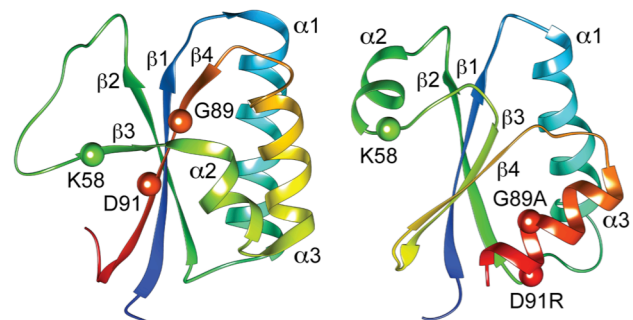


Figure 5. Left-hand side, gsKaiB; right-hand side, fsKaiB. The labeled residues play roles in fold switching. From Chang et al. (2015).

- KaiB fold switching regulates oscillator function and clock output.

When the LiWang lab made mutations that favored the fsKaiB state, clock function was abolished, as observed using in vitro KaiC phosphorylation assays (Chang et al., 2015). They also observed that reporter genes in live cyanobacteria lost circadian rhythmicity when cells harbored these same KaiB mutations (Figure 6). Thus, the slowness of KaiB fold

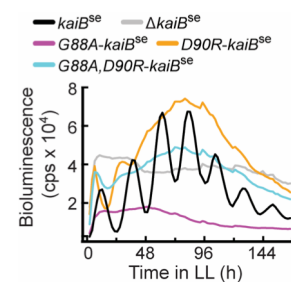


Figure 6. Bioluminescence from cyanobacterial strains carrying a P_{kaiB} *luc* reporter. Cells harbored $kaiB^{se}$, $G88A-kaiB^{se}$, $D90R-kaiB^{se}$, or $G88A,D90R-kaiB^{se}$, or cells with $kaiB^{se}$ deletion. The amino acid substitutions shift the $gsKaiB \rightleftharpoons fsKaiB$ equilibrium to the right. From Chang et al. (2015).

switching is an essential part of the mechanism of the circadian clock of cyanobacteria. In vivo experiments were carried out in collaboration with the Susan Golden lab at UC San Diego.

- KaiB fold switching regulates slow formation of the KaiB-KaiC complex.

The LiWang lab discovered that shifting the gsKaiB \leftrightarrow fsKaiB equilibrium to the right by strategic amino acyl substitutions increases the rate at which KaiB binds KaiC (Figure 7). This finding is significant because it showed that the kinetics of KaiB-KaiC complex formation is dictated by the metamorphic fold-switching properties of KaiB (Chang et al., 2015).

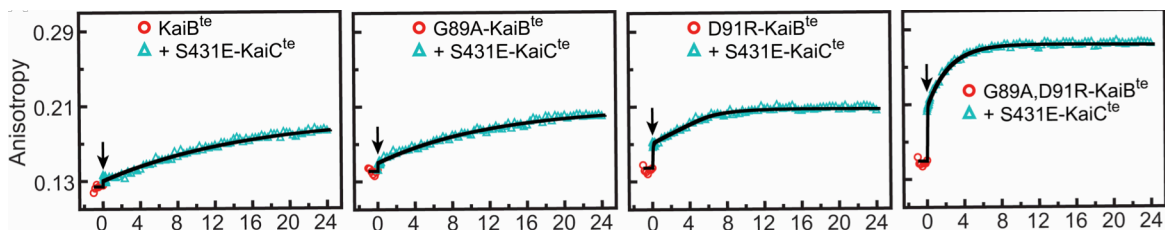


Figure 7. The same KaiB mutants used in the experiments shown in Figure 6 were used in the binding-kinetics experiments shown here. Shifting the gsKaiB \leftrightarrow fsKaiB equilibrium to the right through these amino acyl substitutions increases the rate of KaiB-KaiC binding. From Chang et al. (2015).

- KaiB and SasA bind to similar sites on the CI domain of KaiC.

In order to get a structural foothold on the basis of KaiB-KaiC and SasA-KaiC interactions, and KaiB-SasA competition for KaiC, the LiWang lab calculated EPR-restrained models of KaiB-KaiC and SasA-KaiC structures, in collaboration David Britt at UC Davis. As shown in Figure 8, EPR data suggest that KaiB and SasA bind to similar B-loop regions on the CI domain of KaiC (Chang et al., 2015). This result helped quell the controversy regarding whether KaiB binds to the CI or CII domains of KaiC.

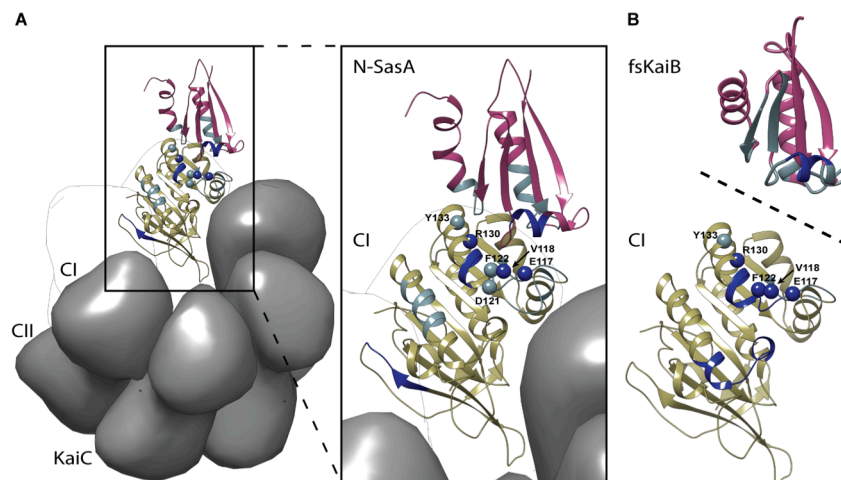


Figure 8. Qualitative models of (A) SasA-KaiC and (B) KaiB-KaiC binding. Sparse EPR data were used as restraints in generating these models. From Chang et al. (2015).

2015 – 2016:

- Fold-switched KaiB binds to the post-hydrolysis state of KaiC.

A major gap in knowledge was the structural basis of the KaiB-KaiC interaction. Several labs starting 20 years ago tried unsuccessfully to crystallize KaiB-KaiC complexes, because the binding affinity was too weak. Labs docked the crystal structure of free KaiB onto the crystal structure of free KaiC using electron microscopy of complexes as a guide (Pattanayek et al., 2013; Pattanayek et al., 2008; Pattanayek et al., 2011; Snijder et al., 2014; Villarreal et al., 2013). These attempts were realized to be misdirected once the LiWang lab discovered that KaiB does not bind KaiC using its crystal structure (gsKaiB), but uses a completely different fold, fsKaiB.

The EPR-based model shown in Figure 8 lacked atomic resolution. Thus, the LiWang lab in collaboration with the lab of Carrie Partch at UC Santa Cruz used KaiB mutants locked in the active fsKaiB state to form stable complexes between KaiB and the CI domain of KaiC. The complex readily crystallized (Figure 9). X-ray diffraction data sets collected on these crystals allowed the structure of the complex between KaiB and the CI domain to be solved to 1.8 Å resolution (Figure 10).

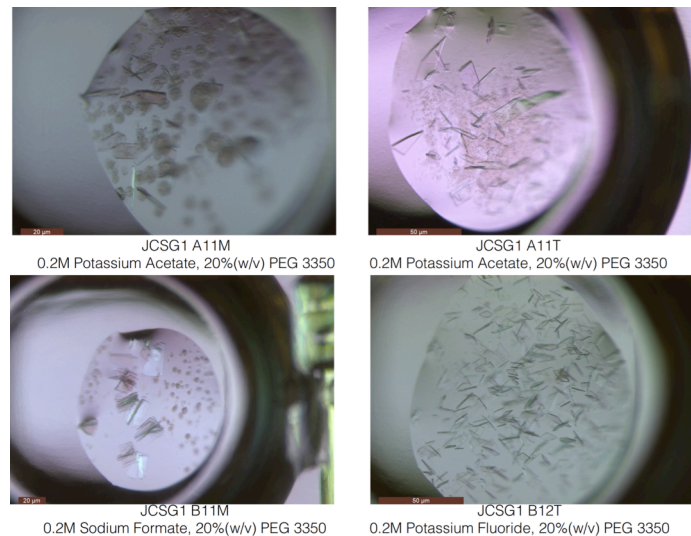


Figure 9. Crystals of KaiB bound to the CI domain of KaiC. X-ray diffraction data was collected on these crystals at the Advanced Light Source at Lawrence Berkeley National Laboratory.

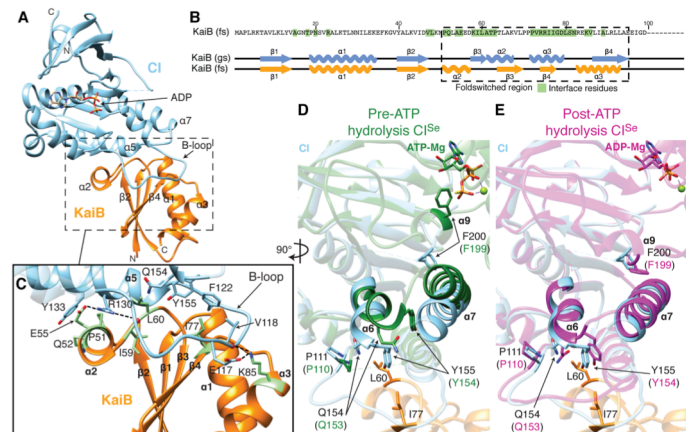


Figure 10. Fold-switched KaiB binds to the post-hydrolysis state of KaiC CI. (A) 1.8 Å crystal structure of the KaiB-CI complex. Orange, KaiB; sky-blue, CI. (B) Secondary structures of gsKaiB (PDB 2QKE) and fsKaiB. Residues of KaiB that interact with CI are highlighted in green. (C) Zoomed-in view of the boxed region in (A). Dashed lines: electrostatic KaiB-CI interactions. (D) Dark green, pre-hydrolysis state of CI. (E) Magenta, post-hydrolysis state of CI (PDB 4TLA, subunit E). (Submitted to *Science*.)

The CI domain of KaiC is an ATPase, and thus has pre-ATP hydrolysis and post-ATP hydrolysis states. Without this ATPase activity, KaiC cannot bind KaiB (Phong et al., 2013), but the basis for this observation was not clear. Recently, it was shown that the two ATPase states have significantly different conformations (Abe et al., 2015). The LiWang/Partch structure revealed that KaiB binds to the post-hydrolysis state of KaiC. This discovery is significant, because it showed that ATP hydrolysis by the CI domain of KaiC is necessary for it to adopt a conformation that allows it to bind KaiB.

- Six KaiB proteins assemble on the CI ring to form a hexameric KaiB-KaiC complex.

The LiWang/Partch team then solved a 3.87 Å-resolution crystal structure of KaiB bound to full-length KaiC, and found that six KaiB proteins bind to the hexameric CI ring (Figure 11). Here, KaiB is bound to the post-hydrolysis state of KaiC, as was also observed in Figure 9. This structure is significant, because it revealed interactions between adjacent KaiB proteins, suggesting that KaiB binds to KaiC cooperatively.

- KaiA forms an autoinhibitory conformation upon sequestration by the KaiB-KaiC complex.

Recall that KaiA stimulates KaiC autophosphorylation during the day, and that KaiA is inhibited by KaiB at night to allow KaiC autodephosphorylation to occur unimpeded. Solving a high-resolution structure that reveals the mechanism of KaiA inactivation was a top priority in the field for almost 20 years. The reason past attempts were unsuccessful was that investigators used wild-type KaiB, which does not bind KaiC with an affinity needed for crystallization.

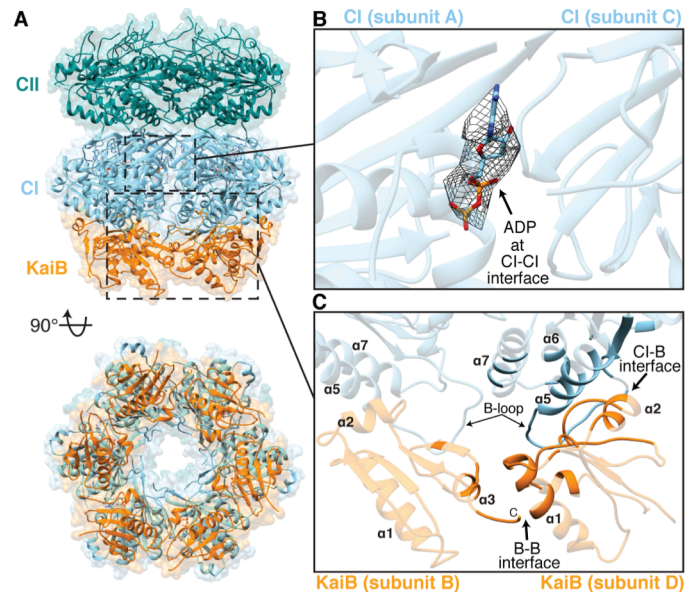


Figure 11. Six KaiB proteins assemble on the CI ring in a hexameric KaiB-KaiC complex. (A) Overview of the KaiB-KaiC hexamer. KaiC includes CII hexamer (Dark cyan) and CI hexamer (sky-blue). Orange, KaiB. (B) Zoomed-in view of the boxed region in (A) showing bound ADP highlighted by mesh representing $F_{obs} - F_{calc}$ omit maps contoured at 2.5σ . (C) Zoomed-in view of the boxed region in (A) showing KaiB interfaces in the KaiB-KaiC hexamer. Interfacial residues are shown in non-transparent mode. (Submitted to *Science*.)

KaiA has two KaiC-binding sites, and the LiWang group found, using binding experiments, that both sites are inactivated by a single KaiB monomer (Tseng et al., 2014). How one KaiB monomer is able to inactivate both sites on KaiA was a mystery until the LiWang/Partch team solved a 2.7 Å crystal structure of a KaiA-KaiB-KaiC complex (Figure 12). The secret sauce to success was using a mutant of KaiB that is locked in its active fsKaiB conformation. The crystal structure revealed that KaiB binds to KaiA in such a way as to induce KaiA to adopt a self-inhibited conformation, in which its KaiC-binding sites are occluded by α -helices that connect its N- and C-terminal domains. The crystal structure also showed that KaiA increases the KaiB-KaiC interfacial surface area over that found in the binary KaiB-KaiC complex, thereby revealing the basis of KaiA-KaiB-KaiC binding cooperativity that the LiWang group reported earlier (Tseng et al., 2014).

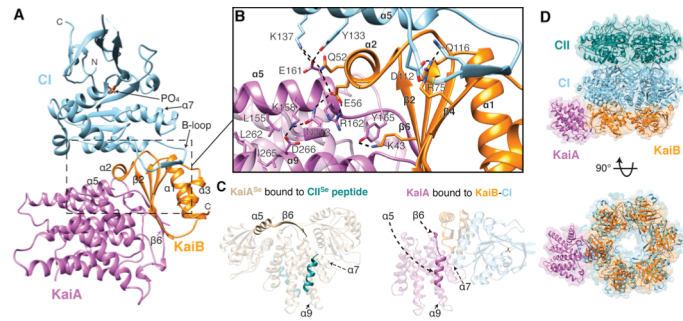


Figure 12. Long-range, allosterically induced auto-inhibition of KaiA in a KaiA-KaiB-KaiC CI complex. (A) The ternary KaiA-KaiB-KaiC CI domain complex. Orange, KaiB; sky-blue, CI; orchid, KaiA. (B) Zoomed-in view of the boxed region in (A). Dashed lines: intermolecular electrostatic interactions. (C) Left: Crystal structure (PDB 5C5E) of KaiA (tan) bound to KaiC CII peptides (dark cyan). Right: The KaiA-KaiB-CI complex, with same coloring scheme as in (A). (D) Superposition of the KaiA-KaiB-KaiC CI structure with that of the KaiB-KaiC hexamer. (Submitted to *Science*.)

- The NMR structure of a CikA-KaiB complex reveals intermolecular interactions that are essential for output signaling.

Recall that the phosphatase CikA is activated by interactions with KaiB at night. Once activated, CikA targets the transcription factor RpaA for dephosphorylation, thereby inactivating it and shutting off expression of clock-controlled genes. It has been a mystery as to how KaiB activates CikA, because there were no high-resolution structures of a CikA-KaiB complex. The trick to making a stable CikA-KaiB complex was to use a mutant of KaiB locked in its active fold-switched state, fsKaiB. Shown in Figure 13 is the LiWang lab's NMR structure of a complex between the pseudo-receiver domain of CikA, ^{PsR}CikA, and KaiB. They discovered CikA-KaiB interactions between highly conserved residues that

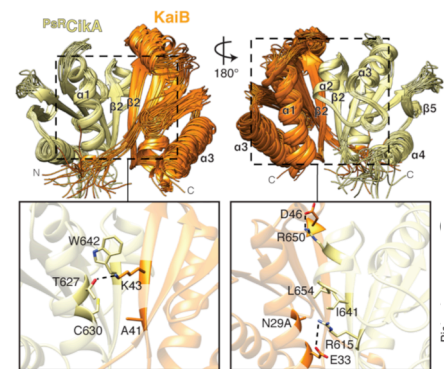


Figure 13. NMR structure of the ^{PsR}CikA-KaiB complex reveals key intermolecular contacts essential for output signaling. Top, ensemble of the 20 lowest-energy NMR structures of the ^{PsR}CikA-KaiB complex. Orange, KaiB; khaki, ^{PsR}CikA. Bottom, zoomed-in views using the average minimized structure from the ensemble. Dashed lines: intermolecular electrostatic interactions. (Submitted to *Science*.)

are critical for CikA activation. Interestingly, CikA binds to the same site on KaiB as KaiA does. Thus, the LiWang group found that CikA and KaiA compete for binding to the KaiB-KaiC complex. This discovery implies that through competition KaiA provides another layer of regulation of CikA. Likewise, CikA adds a layer of regulation of the KaiABC oscillator. Hence, the distinction between oscillator and output pathways are blurring.

- Elucidating clock mechanism using real-time fluorescence spectroscopy.

The biochemistry of life is largely investigated by relating the static structures of proteins to their functions. The static structures of proteins are determined mostly by X-ray diffraction crystallography. This atomic-resolution approach is appropriate for most biological systems. However, biological clocks, by definition, are always moving. The gears of biological circadian clocks are made of proteins that undergo complex motions hour by hour throughout the day and night. Thus, static structures of clock proteins

trapped inside crystals are snapshots of a highly dynamic system. The crystal structures presented above are therefore only part of the story. To fill in the gaps, the LiWang group has started to carry out real-time measurements of reconstituted, fully functional clock systems using different biophysical techniques. Shown in Figure 14 is real-time fluorescence anisotropy data of KaiB and SasA, measured simultaneously. Temporal increases in fluorescence anisotropy indicated when SasA

and KaiB bound to KaiC. As can be seen, SasA binds to KaiC four hours before KaiB binds. The reason for this four-hour delay is because KaiB is impeded by the need to switch from an inactive gsKaiB fold to an active fsKaiB fold. Thus, this experiment reveals that KaiB fold switching provides SasA with a temporal window with which to activate RpaA.

The LiWang group is extending this approach to measure temporal differences between the other clock proteins (KaiA, KaiC, CikA, and RpaA). They are even planning to use a fluorescently labeled DNA oligomer to measure RpaA-DNA binding rhythms in a fully

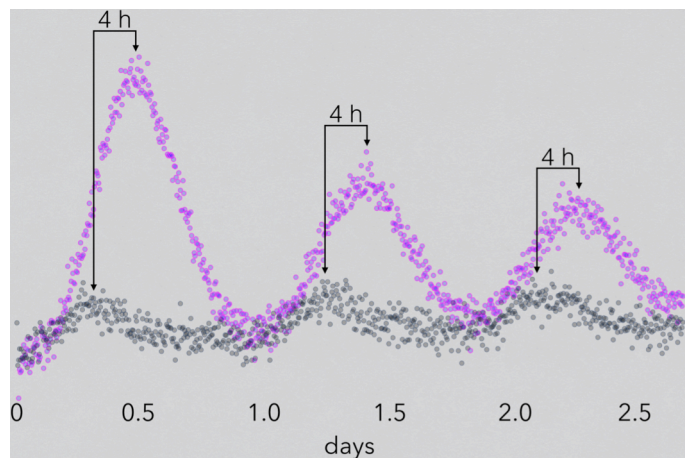


Figure 14. Real-time fluorescence anisotropies of KaiB and SasA, measured simultaneously. An increase in fluorescence anisotropy indicates an increase in molecular size, and therefore reveals the temporal assembly and disassembly of KaiB- and SasA-containing complexes. (Unpublished.)

reconstituted clock reaction. These time profiles will reveal for the first time the complete pathway of transmission of clock signals: oscillator → output → transcription factor → DNA binding. This information is expected to provide much deeper mechanistic insights into the cyanobacterial circadian clock, and allow detailed mathematical modeling.

- Elucidating clock mechanism using real-time NMR spectroscopy.

As shown in Figure 1 (on the first page of this Final Report), the LiWang group is also optimizing real-time measurements of oscillating clock reactions by NMR. The great advantage of NMR is that it provides atomic resolution. As such, real-time NMR is expected to reveal the mechanism of a biological circadian clock at a level of detail that is orders of magnitude greater than is currently achievable. The resulting vertical leap in understanding is anticipated to have far-reaching impacts on the field of chronobiology. For the first time, it will be possible to observe time signals as they ripple across the lengths of circadian clock proteins and are transmitted from one clock protein to the next, cycle after circadian cycle, in real time. The motions of atoms within the proteins will betray their roles in propagating clock signals, and thereby provide deep insights into the mechanism of biological timekeeping. It is expected that the work proposed here will be highly transformative and paradigm shifting.

- Elucidating the KaiB fold-switching pathway.

How KaiB flips between its inactive gsKaiB fold to its active fsKaiB fold remains completely mysterious. For example, does KaiB completely unfold and then refold, or do only parts of it unfold and refold? The LiWang group has started to address this question using the power of NMR to detect protein motions across several different time scales, from picoseconds to hours. The most biological relevant motions often occur on the μ s-ms time scale. Two effective NMR experiments with which to detect such chemical exchange dynamics are the Carr-Purcell-Meiboom-Gill relaxation-dispersion (CPMG RD) experiment (Loria et al., 1999), and the chemical exchange saturation transfer (CEST) experiment (Vallurupalli et al., 2012). The CPMG RD experiment detects chemical-exchange events toward the shorter end of the μ s-ms time scale, whereas the CEST experiment is sensitive to longer ms events.

Shown in Figure 15 are CPMG RD and CEST data from the LiWang lab collected on a sample of uniformly ^{15}N -labeled KaiB. Data for residues G38, Q52, and E56 are shown as representative relaxation profiles. Data is color-coded onto the ribbon structure of KaiB. Residues experiencing shorter μs -ms chemical exchange events are shown in magenta, whereas residues that experience longer ms exchange events are shown in cyan. Although some residues experience chemical exchange on both time scales, especially those located at the dimer interface, others only experience motions on one of the two time scales or on neither time scale.

By using a suite of relaxation experiments, the LiWang lab expects to identify residues playing key roles in $\text{gsKaiB} \rightleftharpoons \text{fsKaiB}$ fold switching, and will use the data to elucidate the fold-switching pathway of KaiB. This knowledge is expected to fill a significant gap in the mechanistic understanding of the cyanobacterial circadian clock.

Because the circadian clocks of all organisms use proteins, the work described here is anticipated to produce new and important insights into the basics of timekeeping that all systems must possess.

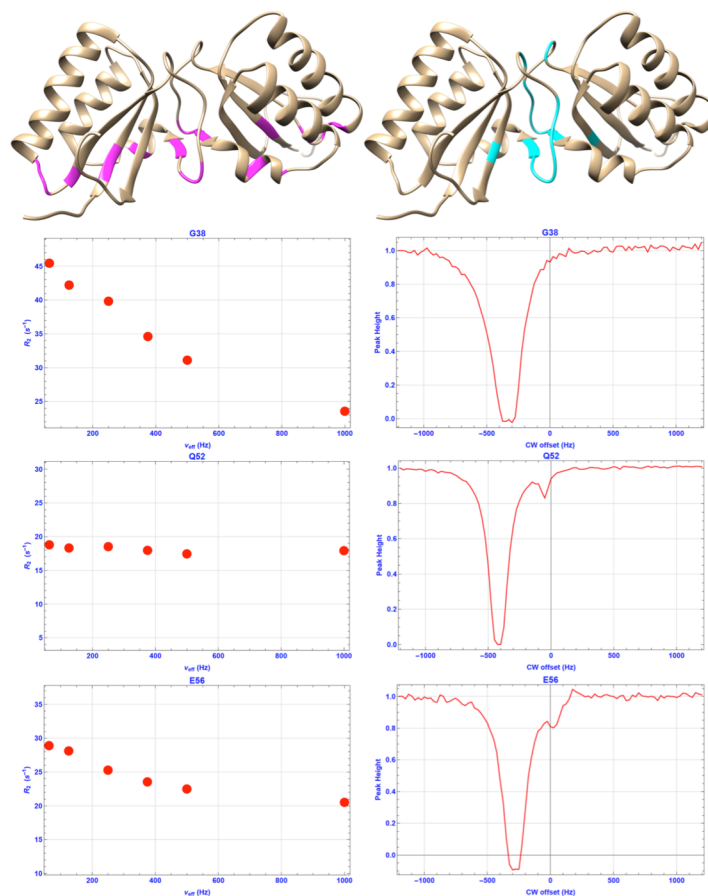


Figure 15. ^{15}N relaxation measurements along the KaiB main chain. The left column shows CPMG RD measurements that are sensitive to μs -ms chemical-exchange events. Regions that were found to experience such motions are colored magenta on the ribbon structure of KaiB (PDB ID: 2QKE). Along the right column are CEST experiments that reveal motions on the longer ms time scale. Residues experiencing these motions are colored cyan on the ribbon structure of KaiB. Relaxation profiles of residues G38, Q52, and E56 are shown as examples of the data. Residue G38 experiences μs -ms motions, but not longer ms motions. Residue Q52 experiences longer ms motions, but not μs -ms motions. Residue E56 experiences both μs -ms and longer ms motions. (Unpublished.)

Peer-reviewed papers describing work supported by AFOSR grant FA9550-13-1-0154:

1. 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.** (2015) A protein fold switch joins the circadian oscillator to clock output in cyanobacteria, *Science*, **349**, 324-328.
2. Roger, T., Chang, Y. -G., Bravo, I., Latham, R., Chaudhary, A., Kuo, N. -W., **LiWang, A.** (2014) Cooperative KaiA-KaiB-KaiC interactions affect KaiB/SasA competition in the circadian clock of cyanobacteria, *J. Mol. Biol.*, **426**, 389-402.

Accolades received for work supported by AFOSR grant FA9550-13-1-0154:

- Mori, T., Mchaourab, H., and Johnson, C. (2015). Circadian Clocks: Unexpected Biochemical Cogs. *Current Biology* 25, R842-R844.
- www.rsc.org/chemistryworld/2015/06/molecular-machinery-behind-circadian-clocks-ticking-revealed
- <http://phys.org/news/2015-06-scientists-decipher-tick-tock-biological-clocks.html>

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Andy LiWang

Program Manager

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Patrick Bradshaw

Reporting Period Start Date

8-1-2013

Reporting Period End Date

7-31-2016

Abstract

The LiWang lab has published two papers under this grant (Chang et al., 2015; Tseng et al., 2014), and has a third paper currently under peer review at the journal Science. In the paper by Tseng et al., undergraduate researchers are authors including one Hispanic undergraduate student. The paper by Chang et al., published in Science, received accolades: (Mori et al., 2015), www.rsc.org/chemistryworld/2015/06/molecular-machinery-behind-circadian-clocks-ticking-revealed, <http://phys.org/news/2015-06-scientists-decipher-tick-tock-biological-clocks.html>. Moreover, this grant has spawned several new directions in circadian-clocks research for the LiWang lab, as will be described in the Research Summary section. The discoveries of the LiWang lab are summarized in a recent YouTube video (https://youtu.be/DcuKifCRx_k). For this animation, the LiWang lab worked closely with the instructor of the undergraduate BioClock Studio course at UC San Diego (<http://ccb.ucsd.edu/the-bioclock-studio/index.html>). There were iterative cycles of animation by undergraduates and suggested edits by the LiWang lab to produce the published video.

Circadian

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Research Objectives

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