UNIVERSITY OF CALIFORNIA, MERCED

Fluorescence monitoring of cyanobacterial circadian clock components *in vitro* elucidates phase relationships, improves period/amplitude effect measurements, and uncovers mechanisms for robustness

A dissertation submitted in partial satisfaction of the requirements
for the degree Doctor of Philosophy

in

Chemistry and Chemical Biology

by

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2020

Dedication

Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. – Marie Curie

In dedication to my incredible grandmother, Beverley Donelson, for inspiring me to work towards helping others above all else.

Acknowledgement

I would like to thank my advisor Dr. Andy LiWang for his support and guidance through this research. My growth as a scientist has been directly proportional to my interactions with both Andy and my other mentors Dr. Yonggang Chang, Dr. Roger Tseng, and Dr. Archana Chavan. Fruitful collaborations and discussions with Dr. Carrie L. Partch, Dr. Susan S. Golden, and Dr. Michael Rust's teams that have helped me to become a more well-rounded scientist. I would like to acknowledge the significant contributions by Dr. Carrie L. Partch and her graduate student Jeffrey A. Swan at UCSC that were instrumental in shaping such a beautiful story supported by rigorous experimentation and analyses. I would also like to thank my committee members, Dr. Victor Muñoz, Dr. Ajay Gopinathan, Dr. Michael Colvin, and Dr. Mike Myers for their mentorship and invaluable feedback.

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Vita

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Education

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Research Experience

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Analyzed biological clock protein-protein interactions *in vitro* by designing novel real-time fluorescence method. Played a key role in optimizing throughput, temporal resolution, sensitivity, and flexibility while substantially reducing labor. Conducted extensive research by coordinating with scientists at UC Merced, UC San Diego, UC Santa Cruz, and the University of Chicago. Explored and uncovered new cooperative relationship between cyanobacterial clock output SasA and core KaiB proteins.

- Performed quantitative analysis of large data sets while allocating period, amplitude, and phase relationships to thousands of self-sustaining oscillations.
- Engineered more than 100 mutant protein constructs, including rational design, DNA mutagenesis and cloning, protein expression, purification, and characterization.
- Published one 1st-author and two coauthor peer-reviewed publications, with one 1st-author manuscript currently submitted to Science.
- Innovative work was utilized to obtain a $125K supplemental grant from NIH to purchase instruments for laboratory.
- Awarded 10 fellowships, four poster competition awards, best lightning talk award, and 12 travel awards for excellent research work.

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Publications

1. **J. Heisler**, J. Swan, J.G. Palacios, C. Sancar, D.C. Ernst, R.K. Spangler, C.R. Bagshaw, S. Tripathi, P. Crosby, S.S. Golden, C.L. Partch, and A. LiWang, Structural Mimicry Confers Robustness in the Cyanobacterial Circadian Clock. 2020, biorxiv.org/content/10.1101/2020.06.17.158394v1 (In Review)
2. G. K. Chow, A. Chavan, **J. Heisler**, YG Chang, A. LiWang, R.D. Britt, Monitoring Protein-Protein Interactions in the Cyanobacterial Circadian Clock in Real Time via Electron Paramagnetic Resonance Spectroscopy. Biochemistry, 2020, doi.org/10.1021/acs.biochem.0c00279
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5. T. Le, A. Qureshi, **J. Heisler**, L. Bryant, J. Shah, T.D. Wolkow, and R. Pyati, Polycyclic Aromatic Hydrocarbons and Small Related Molecules: Effects on Schizosaccharomyces pombe Morphology Measured by Imaging Flow Cytometry. Journal of Yeast and Fungal Research, 2014, 5(6), 84-91; doi.org/10.5897/JYFR2014.0137

6. **J. Heisler**, L. Elvir, F. Barnouti, E. Charles, T.D. Wolkow, and R. Pyati, Morphological Effects of Natural Products on Schizosaccharomyces pombe Measured by Imaging Flow Cytometry. Natural Products and Bioprospecting, 2014, 4, 27-35; doi.org/10.1007/s13659-014-0004-8

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- UC Merced Graduate Dean's Dissertation, **Fellowship** (2019)
- UC Merced Chemistry and Chemical Biology Dissertation, **Fellowship** (2019)
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- AAAS-NSF Emerging Researchers National Conference in STEM, **Selected and Travel Award** (2019)
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- 255th Annual American Chemical Society National Meeting in New Orleans, Poster Presentation (2018)

- Center for Circadian Biology Workshop at UC San Diego, **Invited Speaker** (2017)
- University of California Board of Trustees Meeting, **Invited Speaker** (2017)
- GradSlam Competitions at UC Merced, https://tinyurl.com/sul996n **Top 10 Finalist** (2017 and 2016)
- Center for Circadian Biology Symposium at UC San Diego, **Outstanding Poster Award** (2017)
- Quantitative Systems Biology Retreat at UC Merced, **Best Lightning Talk** (2016)
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- University of North Florida Poster Session in Jacksonville, **1st Place** (2012)

Abstract

The rising and setting of the sun, and the environmental changes in light and temperature that are associated with this rhythm, has shaped nearly all life on Earth. Biological clocks that adapt organism's metabolism, physiology, and behavior to best take advantage of this circadian cycle have heavily influenced the evolution of plants, animals, and bacteria. Despite the importance of biological clocks, no clock mechanism is well understood at the molecular level. A model system that is uniquely suited for detailed mechanistic studies has been established in cyanobacteria, as it's oscillator is composed of only three core proteins, KaiA, KaiB, and KaiC, and three output proteins SasA, CikA, and RpaA, and can be reconstituted *in vitro*. Here, real-time fluorescence spectroscopy has been utilized to assign phase relationships, measure period and amplitude effects from modulating core and output protein concentrations, and uncovered a paradigm breaking dose-dependent compensation for low KaiB concentrations by SasA.

Joel Curtis Heisler

Chemistry & Chemical Biology, Ph.D.

University of California, Merced, 2020

Committee Chairman: Professor Victor Munoz

Chapter I

Introduction to the cyanobacterial clock

Significance of biological clocks

Life in every kingdom has evolved an endogenous molecular mechanism, termed circadian (~24-h) clock. The physiology and behavior of organisms synchronize with the daily environmental changes such as light and temperature caused by Earth's rotation. Circadian clocks have been demonstrated to confer fitness advantage, and in humans, they have been implicated in cancer, aging, sleep, neurodegeneration, and immunity.[1–10] Many drugs in the top- 100 best-seller list target circadian genes in the kidney, lung, brainstem, aorta, adrenal gland, skeletal muscle, brown fat, white fat, cerebellum, heart, liver, and hypothalamus.[11] However, the detailed mechanism of how biological clocks generate 24-h rhythms remains elusive. Not only is the molecular timekeeping, or input, an unresolved scientific question, but the transduction of the clock signal from the clock to the rest of the cell is also a poorly understood and highly complex. This signal propagation brings about changes in organisms to better adapt to predictable changes associated with day/night transitions and is explored here.

Elucidating the inner workings of circadian clocks, which are fundamental to all life forms, is a difficult problem due to the vast complexity exhibited in these clock systems.[12–16] One example of this complexity is the presence of multiple distinct oscillators in cells and tissues contributing to circadian timing, including free-running rhythms with different period lengths in the same organism.[17,18] There are many interlocking feedback loops that allow organisms to have robust circadian rhythms capable of entraining to environmental stimulus, yet the basic nature of endogenous timekeeping is very poorly understood.

Initial efforts to gain insight into biological clocks utilized genetic screens for mutations that affected circadian rhythmicity in insects, fungi, and plants.[19–21] Additional work done with the *Drosophila* model showed that posttranslational regulation was critical for eukaryotic clock system rhythmicity by constitutively inducing known clock genes *period* and *timeless*, which resulted in cycling in coding protein abundances.[22] Eventually cyanobacteria were recognized as exhibiting circadian behaviors that are fundamentally the same as the circadian rhythms in eukaryotes, and because of their relative simplicity when compared to all other biological clocks have become a prominent model organism.[23,24] In particular, *Synechococcus* sp. strain PCC 7942 was ideal because it can be transformed by circular or linear DNA, can receive DNA by conjugation, and can express reporter genes.[25,26]

Cyanobacterial circadian oscillator

A biological clock at the core is simply a collection of highly specialized proteins that produce a synchronized rhythm of time keeping. To be classified as a true biological clock, the circadian system must satisfy the following criteria; (*i*) endogenous timekeeping independent of outside stimuli, often referred to as a free running clock, (*ii*) temperature compensation within physiologically relevant conditions for a given organism, and (*iii*) entrainment such that the biological clock can adjust to external environmental cues, for example the presence or absence of light.[27] The most simplistic *bona fide* biological clock, or circadian oscillator, with each of these characteristics is found in cyanobacteria, a photoautotrophic bacteria which has become an ideal model organism for detailed mechanistic studies of molecular timekeeping.[24,28–32] Interestingly, a large portion of the cyanobacterial genome is rhythmically expressed, which has been aptly called a circadian orchestration of gene expression.[33,34] Critically, cyanobacteria have been found to synthesize ATP under light exposure while ADP levels rise in darkness.[35–37] Capitalizing on these known metabolic transitions, cyanobacterial biological clock system entrainment to exogenous ques has been simulated *in vitro* by altering ATP/ADP ratios, which produced significant phase shifts in molecular timekeeping, consistent with bioluminescence studies monitoring rhythms *in vivo*.[38] Further work has elucidated circadian clock entrainment to pulses of oxidized quninones, present in onset of darkness, that reset the clock system both *in vitro* and *in vivo*.[39]

Circadian rhythm is ultimately derived from a near 24-h transcriptional and translational oscillatory process (TTO) in which expressed clock proteins result in

a negative feedback regulation of clock genes.[40,41] It was thought that a transcription-translation feedback loop was a mandatory component of any circadian oscillator until the cyanobacterial biological clock was shown to keep time independent of *de novo* transcription and translation processes, demonstrating its post-translational nature.[32] This discovery set the cyanobacterial model system apart from all others, such that the core components KaiA, KaiB, and KaiC can be reconstituted *in vitro*, allowing unprecedented experimental control and analysis.[31] Moreover, recent findings confirm that the post translational oscillator (PTO) is sufficient to generate oscillations *in vivo*, revealing that growing cyanobacteria do not require transcription-translational feedback loops (TTFLs) to be successful.[42–44] Molecular timekeeping in *Drosophila*, *Arabidopsis*, *Neurospora*, and mammals are all products of phosphorylation of core clock proteins; implying a deeper understanding of the bacterial clock can be applied to better understand more complex eukaryote clock systems.[40] Insight gained through mechanistic studies from the isolated cyanobacterial PTO *in vitro* therefore has far reaching implications.

As previously stated, the cyanobacterial biological clock core oscillator components consist of only three proteins, KaiA, KaiB, and KaiC (*kai* meaning “cycle” in Japanese), and can be reconstituted *in vitro* with ATP as an energy source.[31] These core proteins produce a near 24-h rhythm of protein-protein binding interactions leading to temperature compensated molecular timekeeping independent of light exposure or other external stimuli, described in this chapter. Importantly, this rhythm is independent of external stimuli, but can indeed be entrained by input. Among the three core oscillator proteins, KaiC is the heart of the clock, displaying an approximately 24-h rhythm of auto-phosphorylation and auto-dephosphorylation, with KaiA, the positive regulator, promoting the former, and KaiB, the negative regulator, promoting the latter by inhibiting KaiA.[45–47] Unique to the cyanobacterial clock system, the structures of each of the individual components have been determined (Figure 1.1).[48–51] Intra- and inter-molecular mechanisms of the core oscillator components KaiC, KaiA, and KaiB work in synchrony to regulate the timing of ATP hydrolysis with KaiC subunits, resulting in the synchronized near 24-h KaiC phosphorylation cycle.

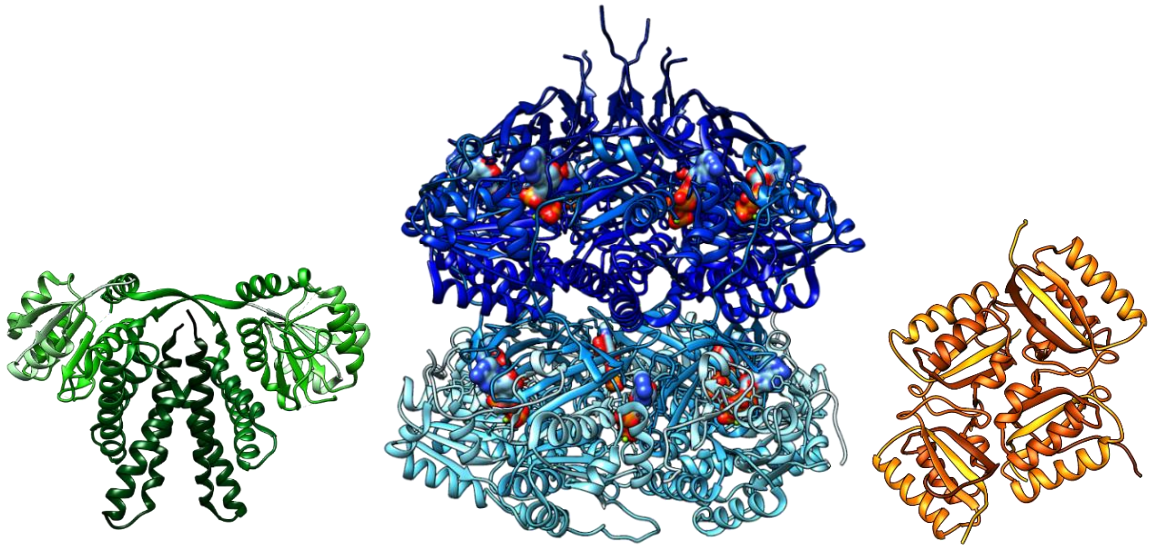

Figure 1.1. Structural depictions of KaiA, KaiB, and KaiC. UCSF Chimera structural models of KaiA (PDB:1R8J), KaiC (PDB:4O0M), and KaiB (PDB:2QKE). ATP molecules are represented by spheres with (red) oxygen and (orange) phosphate groups, oriented between the (middle) KaiC hexamer subunits.

Core oscillator components

KaiA : 284 residues, 32.61 kDa

VLSQIAICIWVESTAILQDCQRALSADRYQLQVCESGEMLLEYAQTHRDQIDCLI
LVAANPSFRAVVQQLCFEGVVVPAIVVGDRDSEDPDEPAKEQLYHSAELHLGIH
QLEQLPYQVDAALAEFLRLAPVETMADHIMLMGANHDPELSSQQRDLAQRLQE
RLGYLGVYYKRDPDRFLRNLPAYESQKLHQAMQTSYREIVLSYFSPNSNLNQSI
DNFVNMAFFADVPVTKVVEIHMELMDEFAKKLRVEGRSEDILLDYRLTLIDVIAH
LCEMYRRSIPRET

KaiC-FLAG: 527 residues, 59.05 kDa

DYKDDDDKMTSAEMTSPNNNSEHQAIAKMRTMIEGFDDISHGGLPIGRSTLVS
GTSGTGKTLFSIQFLYNGIIEFDEPGVFVTFEETPQDIIKNARSFGWDLAKLVDE
GKLFILDASPDPEGQEVVGGFDLSALIERINYAIQKYRARRVSIDSVTSVFQQYD
ASSVVRRELFRLVARLKQIGATTVMTTERIEEYGPIARYGVEEFVSDNVVILRNV
LEGERRRRTLEILKLRGTSHMKGEYPFTITDHGINIFPLGAMRLTQRSSNVRVSS
GVVRLDEMCGGGFFKDSIILATGATGTGKTLLVSRFVENACANKERAILFAYEES

RAQLLRNAYSWGMDFEEMERQNLLKIVCAYPESAGLEDHLQIIKSEINDFKPARI
AIDSLSALARGVSNNAFRQFVIGVTGYAKQEEITGLFTNTSDQFMGAHSITDSHI
STITDTIILLQYVEIRGEMSRAINVFKMRGSWHDKAIREFMISDKGPDIKDSFRNF
ERIISGSPTRITVDEKSELSRIVRGVQEKGPES

KaiB-FLAG: 110 residues, 12.41 kDa

MSPRKTYILKLYVAGNTPNSVRALKTLKNILEVEFQGVYALKVIDVLKNPQLAEE
DKILATPTLAKVLPLPVRRIIGDLSDREKVLIGLDLLYGELQDSDDFDYKDDDDK

Table 1.1. Sequence analysis of KaiA, KaiB, and KaiC.

	KaiA		KaiB-FLAG		FLAG-KaiC	
Pattern:	Number	Percent	Number	Percent	Number	Percent
A	24	8	6	5	33	6
C	6	2	0	0	3	1
D	20	7	13	12	32	6
E	23	8	6	5	43	8
F	9	3	2	2	28	5
G	8	3	5	5	42	8
H	9	3	0	0	8	2
I	16	6	7	6	47	9
K	6	2	11	10	24	5
L	35	12	18	16	37	7
M	9	3	1	1	14	3
N	8	3	4	4	20	4
P	12	4	6	5	14	3
Q	21	7	3	3	17	3
R	19	7	5	5	40	8
S	17	6	4	4	43	8
T	7	2	5	5	33	6
V	22	8	9	8	33	6
W	1	0	0	0	3	1
Y	12	4	5	5	13	2
Aliphatic I, L, V	73	26	34	31	117	22
Aromatics F, W, Y	22	8	7	6	44	8
Positive K, R, H	34	12	16	15	72	14
Negative D, E	43	15	19	17	75	14
Tiny G, A, S	49	17	15	14	118	22
Total	284	100	110	100	527	100

KaiA role in the biological clock

The positive element of the core circadian oscillator, KaiA has a primary role of initiating KaiC autophosphorylation through binding the C-terminal tail, known as the A-loop, (residues D500-S519) of KaiC.[46,50,52,53] This activity is contrary to KaiB effect on KaiC and is often referred to as inhibiting KaiC dephosphorylation.

KaiA is a domain-swapped homodimer with two defined domains, a pseudo-receiver N-terminal domain and a novel helical bundle C-terminal domain, connected by a short linker (Figure 1.1).[50,54] There are two distinct conformations that KaiA adopts, an active state with the KaiA-KaiC binding interface located in the C-terminal domain, and the inactive state in which the N-terminal domain is positioned at the KaiC binding site autoinhibiting KaiA-KaiC complex formation.[55,56] The inactive state occurs during the subjective night; KaiB sequesters KaiA from the C-terminal A-loops of KaiC and recruits it to the N-terminal domain of KaiC where it forms a ternary KaiABC complex, known as the recessive nighttime complex, capturing KaiA in the inactive state.[55,56] Binding of KaiA to KaiC has been shown to facilitate nucleotide exchange by widening the KaiC subunit interface, which may play a role in maintaining circadian time.[57] External stimuli sensing is achieved through oxidized quinones inhibiting KaiA binding to KaiC while reduced quinones do not effect KaiC interactions.[39] Lastly, KaiA is also involved in competition with a sensor histidine kinase CikA, such that they share a binding interface on KaiB when the KaiB-KaiC nighttime complex is present. This competition regulates CikA phosphatase activity towards master regulator, RpaA, transcription factor thereby contributing to clock timing propagation throughout the cyanobacterial cell.[34,56,58]

KaiC role in the biological clock

As early as 2004, it was shown that KaiC primarily coordinates genome wide gene expression, including regulating its own expression through a negative feedback loop. Namely, high levels of KaiC *in vivo* repress co-expression through *kaiBC* in addition to repression of rhythmic components of all genes in the cyanobacteria genome.[30] The largest of the three core oscillator proteins, KaiC, is a hexamer with each subunit consisting of two domains, the N-terminal CI and C-terminal CII, derived from a RecA-like gene duplication event, forming highly similar stacked CI and CII ring structure.[59–62] KaiC is part of the AAA+ superfamily and has auto-kinase, auto-phosphatase, and ATPase activity that collectively play an essential role in cyanobacterial timekeeping.[45,63,64] Each ring has six subunit interfaces with ATP binding sites, for a total of twelve bound nucleotides per KaiC hexamer.[49] In addition to ATP binding sites, both CI and CII rings possess Walker's motif A and two putative catalytic carboxylate glutamate residues for phosphorylation.[23] The unique dynamic activities of KaiC make it an intriguing target for structural biologists, mathematical modelers, biophysicists, and geneticists alike.

The first insight into how timing was stored within the core oscillator came through the discovery that KaiC contains two DXXG motifs that are highly conserved in the GTPase superfamily. It was suggested that KaiC was likely autophosphorylated at serine and/or threonine residues due to phosphoserine and phosphothreonine being base labile, allowing for reversible phosphate binding and dissociation.[45,65] Soon thereafter, Ser431 and Thr432 residues were identified by mass spectrometry, presenting four KaiC phosphorylation forms – ST (unphosphorylated), SpT (threonine phosphorylated), pSpT (doubly phosphorylated), and pST (serine phosphorylated).[66] The four phosphorylation states of KaiC are unidirectional with autophosphorylation occurring during the subjective day and autodephosphorylation taking place at subjective night.[64,67–69] Throughout a daily cycle, KaiC ATPase activity hydrolyzes around 15 ATP molecules, which has been shown to be a temperature compensated mechanism.[64] Regardless of overall ATP hydrolysis, it has been demonstrated by multiple groups that KaiC seems to regenerate ATP from ADP as it dephosphorylates CII ring residues, which was demonstrated to be via reversal of the phosphorylation reaction, unlike conventional phosphatases.[70–72] It has also been proposed that the slowness of the clock arises from sequestration of a lytic water molecule in an unfavorable position coupled to rearrangements of the CI ring.[73,74]

KaiC undergoes autophosphorylation while in complex with KaiA during subjective day and autodephosphorylation when interacting with KaiB during subjective night.[75] The day/night transitions are accompanied by significant conformational changes in KaiC; the CI and CII domains are unstacked during the subjective day resulting from a flexible CII domain, however in subjective night phosphorylated Ser431 induces CII rigidity and stacks with CI.[76] Another key discovery regarding KaiC intramolecular interactions was that of monomer subunit shuffling *in vivo* and *in vitro*. KaiC has the ability to exchange subunits of different phosphorylation states which primarily occurs during the subjective night due to KaiA autophosphorylation activity during the day inhibiting monomer shuffling.[77–79] Critically, KaiC subunit exchange and cluster formation at night allowed Emberly and Wingreen to derive an hourglass model to explain Kai oscillator synchronization.[80] Overall, the central piece of the cyanobacterial circadian oscillator, KaiC, is incredibly dynamic with changes in flexibility/rigidity of the CII domain, burying and unburying of A- and B-loops, rhythmic unidirectional phosphorylation of Ser431 and Thr432 residues, and temporally regulated subunit shuffling.

KaiB role in the biological clock

KaiB is the negative element of the core circadian oscillator. The formation of KaiB-KaiC recessive complex constitutes six monomeric KaiB subunits bound to hexameric KaiC.[55,56] KaiB binds to a disordered region in the N-terminal CI domain of KaiC, termed the B-loop, and causes autodephosphorylation of KaiC.[81] Previous work has identified KaiB as a homo-tetramer of two homo-dimers that exists in an equilibrium between tetramer, dimer, and monomer. [48,82,83] The tetramer, dimer, and monomer equilibrium can be manipulated by decreasing temperature and mutant truncations to favor lower oligomerization states.[82] Identifying how KaiB interacted with the other clock proteins KaiC and KaiA was a difficult and controversial undertaking, with some groups suggesting KaiB binds CI and others convinced KaiB-KaiC complex formation occurred on the CII domain of KaiC. Initial inferences elucidated interactions between all three core oscillator Kai proteins, suggesting the formation of ternary KaiABC complex which was shown to be independent of KaiC A-loops.[42] Critically, two years later, the LiWang lab confirmed that KaiB binds to the CI domain of hexameric KaiC and requires ADP bound between KaiC subunits, known as the post-hydrolysis state, to expose the KaiB binding site, named B-loop.[47]

A startling discovery in 2015 showed KaiB is a metamorphic protein; demonstrating that it adopts two distinct folds, one novel tetrameric ground-state inactive fold and a rare fold-switched thioredoxin-like monomer conformation, in which it interacts with other circadian clock components.[84] This metamorphic activity is a key mechanism responsible for cyanobacterial timekeeping, such that the active fold-switched state of KaiB (fsKaiB) is stabilized only when hyperphosphorylated KaiC is present, and after the population of KaiC is autodephosphorylated the fsKaiB dissociates and returns to ground-state (gsKaiB) tetramer.[56,84] Findings that KaiB ring formation on the CI ring of KaiC is cooperative via mass spectrometry-based structural model and mutational analysis is consistent with initial seeding of one fsKaiB monomer to KaiC facilitates recruitment of other KaiB molecules to adjacent subunits of KaiC until a full $KaiB_6$-$KaiC_6$ complex is formed.[83,85]

Propagation of circadian timing from the core oscillator system to the cyanobacterial cell is directly linked to KaiB complex formations. Critically, the KaiB-KaiC binary complex regulates the two component antagonist histidine kinase pair SasA and CikA, which act as a kinase and phosphatase towards master gene regulator RpaA, respectively.[34,86–88] KaiB competes with SasA, for

binding to B-loops of hexameric KaiC CI ring thereby restricting kinase activity and downstream signaling.[81,89] Conversely, CikA phosphatase activity is positively controlled such that its activity is initiated by interacting with the binary KaiB-KaiC nighttime complex.[56,58]

KaiA-KaiC daytime complex

The absolute amount of core clock proteins in a single cell have been shown to be ~500 KaiA, ~20,000 KaiB, and ~10,000 KaiC.[75] However, despite there being more KaiB present than the other two proteins in the cell, it is not involved in the daytime complex. During subjective dawn, KaiC is in a hypophosphorylated state with the CII domain exposing the A-loops, presenting a binding site for the C-terminal domain of KaiA, shown in Figure 1.2.[46,53,54,90]

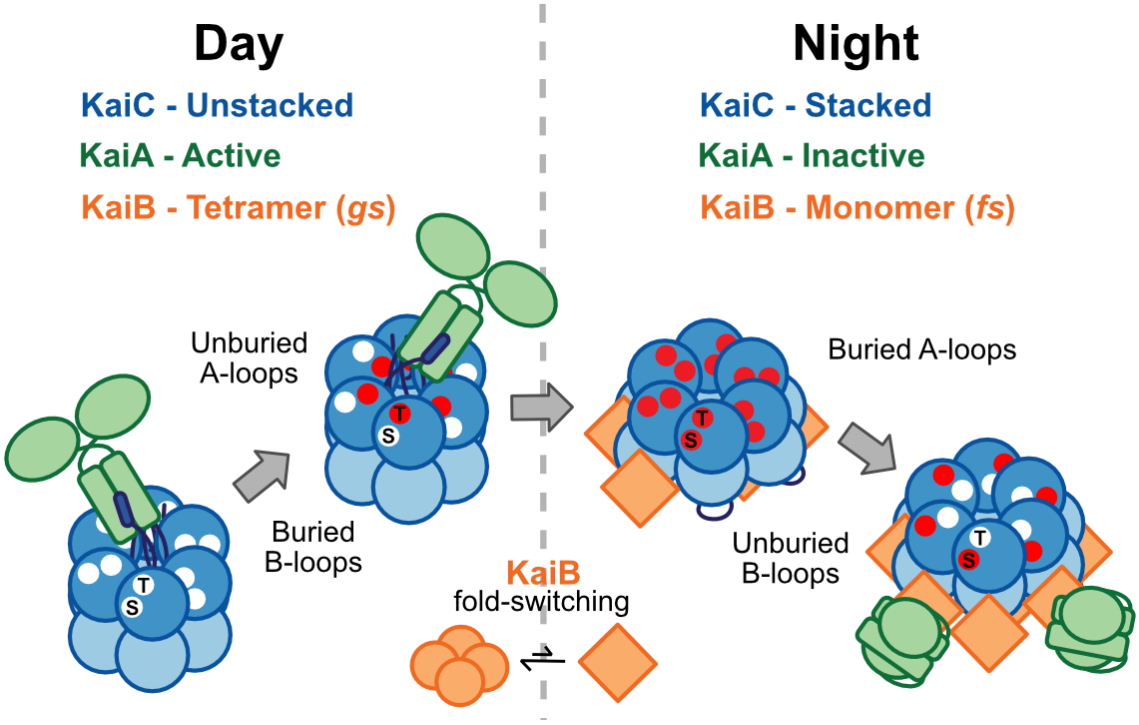

Figure 1.2. Cartoon representations of KaiA, KaiB, and KaiC highlighting day/night transitions. Ordered phosphorylation of (blue) KaiC at Ser431 and Thr432 is shown with (white) non-phosphorylated and (red) phosphorylated residues. Daytime binding of (green) KaiA to exposed A-loops leads to autophosphorylation of unstacked KaiC. Transitioning to nighttime complex formations is coordinated with (orange) KaiB fold-switching and

hyperphosphorylated KaiC, leading to KaiC ring stacking and KaiB binding exposed B-loops. Lastly, ternary complex formations KaiA-KaiB-KaiC highlight recruitment of inactive KaiA to the nighttime recessive complex.

In this orientation, the KaiC CII domain is unstacked from the CI domain, with six ATP molecules bound within the subunits of both CI and CII.[47] The exposed A-loops being stabilized through KaiA binding allow for auto kinase activity in the CII domain of KaiC. This process transfers a gamma phosphate group from bound ATP to KaiC residues Threonine 432 and Serine 431 sequentially on the time scale of hours (ST, SpT, pSpT, pST).[66,69,49,91,45] This phosphorylation rhythm can be monitored *in vitro*, capitalizing on the ability to differentiate between phosphorylated (P-KaiC) and non-phosphorylated (NP-KaiC) molecules, using gel electrophoresis (Figure 1.3). Although this technique was first applied in 2005, it has remained the prominent approach used to study the cyanobacterial clock *in vitro*.[31]

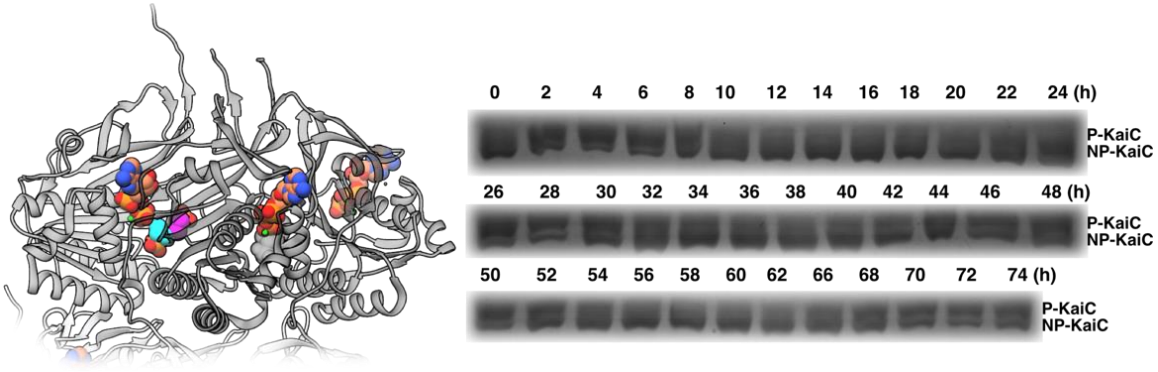

Figure 1.3. KaiC phosphorylation rhythm can be monitored *in vitro*. On left, UCSF Chimera illustration of KaiC C-terminal ring (grey), with ATP molecules at subunit interfaces colored by heteroatoms, magnesium atoms (green), and highlighted phosphorylation-site residues pS431 (pink), pT432 (teal). On right, two-hour aliquots collected from an *in vitro* KaiABC reaction analyzed by SDS-PAGE gel electrophoresis.

Active KaiA binding to the A-loops stabilizes the unburied conformation, promoting KaiC autophosphorylation activity during the subjective day.[50,52,92] An important conformational consideration for KaiC is that increased flexibility in the CII domain is possible due to a more rigid pre-hydrolysis CI domain.[76] Synchronization of the entire population of KaiC phosphorylation states is essential for robust circadian rhythm and is facilitated by a decrease in KaiA affinity for KaiC A-loop binding to hyperphosphorylated KaiC.[75,80] This results in

KaiA preferentially binding to unphosphorylated KaiC subunits, maintaining a coordinated rhythm, which has been termed the Langmuir model. This phenomenon provides a mechanism for ~ten thousand KaiC molecules to undergo a synchronized, near 24-hr, phosphorylation rhythm *in vitro*.[75] Of course, not all KaiC subunits become fully phosphorylated at any giving time, in fact it has been shown time and time again that the maximum phosphorylated proportion of KaiC is around 80% in an *in vitro* oscillating reaction.

KaiB-KaiC-KaiA nighttime complex

The transition from cyanobacterial circadian day complexes to night complexes is brought about by hyperphosphorylated KaiC at both Threonine 432 and Serine 431 (pSpT). With this phosphorylation state, multiple events take place in a currently undefined order. (*i*) ATPase activity in KaiC CI domain yields a post-hydrolysis conformation with ADP molecules bound between the subunits resulting in CII and CI ring stacking and B-loop exposure, (*ii*) active KaiA dissociates from KaiC as the hyperphosphorylated CII domain buries the A-loops providing increased rigidity to CII ring, allowing for CI ring flexibility, (*iii*) the exposed B-loops of KaiC are bound by KaiB that cooperatively form a six monomer ring with the CI ring, and (*iv*) sequestered KaiA dimers bind the KaiB-KaiC complex forming a ternary nighttime complex.[42,47,55,56,76,83] The sequestration of active KaiA away from KaiC A-loops and subsequent recruitment to KaiB-KaiC complex causes a dramatic conformational change in KaiA into an autoinhibitory inactive state in which the N-terminal domains of the asymmetric homodimer tuck into the C-terminal alpha helix bundle concealing its own KaiC binding interface.[56] . This conformational change and ternary complex formation accomplish two goals, it strengthens the KaiB-KaiC complex while simultaneously inhibiting KaiA effect on KaiC phosphorylation throughout the subjective night. During this time, phosphotransferase activity in CII domain of KaiC passes phosphate groups from Threonine 432 and Serine 431 sequentially through ATP synthesis.[67,69] Thus, KaiB inhibits KaiA causing KaiC autodephosphorylation.

Throughout the subjective night, KaiC hexamers undergo subunit exchange which is believed to help maintain synchrony.[67,78,93] Once KaiC is fully dephosphorylated another series of events take place in an undefined order. (*i*) KaiA-KaiB-KaiC ternary complexes dissociate altering KaiA conformation from inactive autoinhibitory state to an active free state while active fold-switched KaiB monomers bound to KaiC dissociate and quickly return to an equilibrium of

predominately inactive ground state tetramers, (*ii*) KaiC CI domains begin to switch from post-hydrolysis state with ADP molecules bound between the subunits into a pre-hydrolysis ATP-bound state, (*iii*) the CI and CII domains of KaiC become unstacked, and (*iv*) KaiC A-loops return to subjective day time unburied conformation with ATP molecules bound between CII subunits.[47,56,84]

Extended oscillator components

RpaA: 249 residues, 28.47 kDa

MKPRILVIDDDSAILELVAVNLEMSGYDVRKAEDGIKGQALAVQLVPDLIMLDLM
LPRVDGFTVCQRLRRDERTAEIPVLMLTALGQTQDKVEGFNAGADDYLTKPFE
VEEMLARVRALLQRTDRIPHAARHSEILSYGPLTLIPERFEAIWFNRTVKLTHLEF
ELLHCLLQRHGQTVAPSEILKEVWGYDPDDDIETIRVHIRHLRTKLEPDPRHPRY
IKTVYGAGYCLELPAETELHQHADQFPSAS

SasA-FLAG: 396 residues, 44.32 kDa

MGESLSPQALAQPLLLQLFVDTRPLSQHIVQRVKNILAAVEATVPISLQVINVAD
QPQLVEYYRLVVTPALVKIGPGSRQVLSGIDLTDQLANQLPQWLVQQEAFFAD
REPPEVNIPFTELGQPETPALQQADAFFQLQQQYADLSERTKFLEQVIALVAHD
LRNPLTAALLAVDTIQIRSQSFSVATAKEMQGLCSLFDQARSQLREIERMIAEILE
ATRHSGESLRINPREVVFEPLLQQVLEQLHERWRSKQQQLITDVPGDLPTLYAD
PDRLRQVLVNLLDNAIKYTPPGGTITIAALHRTSQKVQISISDTGSGIPRDQLSVIF
KNLVRLSRDSSQEGYGIGLSVCQRIVQAHFGRIWVASELGQGSTFHFTMPVYR
YTMPCDYKDDDDK

FLAG-CikA: 761 residues, 85.3 kDa

DYKDDDDKMLAPSSNCSLASQRLTPEGFAQLQSALQDFVATLPQAFYWDSRS
LHTHLRTQTGDCAIAIAAGFQLLLLGRTAAEYCQPHPLSEPHHVSVQFGADSIQ
RYCQATNLPVEYQPALAQLGDLSLNPDLISQFSNLLIAAIAADRAPLAAQYPAVS
VCQPLEQALHWQEEQDRLISQVSAQIRLSLDLSEILTTTIREIRQLLNADRAIIYQF
KPCLDAGLDQRWPLYIPSQSYITYEDRRNEALLSVIDPLVQPGLLITTEEWQRFQ
QGETLLIDSVGFYKERLPEQYSFYERVQVRSVCKIPILVQGRIWGLLVAHQCQQ
DHRWQPRERDILQHLAEHLSIAIYQAQLYGQLQDQTQTLENRVLERTQELIDAL
ALAQAANAAKGEFLATMSHELRTPLTCVIGMSSTLLRWAFGPLTERQREYIKAIH
DSGEHLLELINDILDLSQIEAGKAALQVRPFSLSRLATQTLNTLQEKARLGEIQLM

LDLQLNNRVDVFRADPKRLRQILINLLSNAVKFTEPQGTVFLRVWREGDRAIFQ
VSDTGIGIPESEQAQLFQKFQQLDTSIRRQYGGTGLGLALTKQLVELHGGHIQIE
STVGQGSTFTVWIPEQTLIEPVEPRPSIDNLPAGHILLLEEEDEAATVVCEMLTA
AGFKVIWLVDGSTALDQLDLLQPIVILMAWPPPDQSCLLLLQHLREHQADPHPP
LVLFLGEPPVDPLLTAQASAILSKPLDPQLLLTTLQGLCPPNLSEGDRPSS

Table 1.2. Sequence analysis of SasA, CikA, and RpaA.

	RpaA		SasA-FLAG		FLAG-CikA	
Pattern:	Number	Percent	Number	Percent	Number	Percent
A	19	8	30	8	65	9
C	3	1	3	1	12	2
D	19	8	23	6	43	6
E	21	8	22	6	48	6
F	7	3	14	4	22	3
G	12	5	18	5	37	5
H	10	4	7	2	19	2
I	15	6	25	6	48	6
K	9	4	10	3	15	2
L	33	13	49	12	118	16
M	6	2	5	1	6	1
N	3	1	9	2	16	2
P	15	6	24	6	46	6
Q	10	4	41	10	73	10
R	20	8	25	6	43	6
S	7	3	26	7	46	6
T	14	6	21	5	41	5
V	17	7	31	8	35	5
W	2	1	3	1	11	1
Y	7	3	9	2	17	2
Aliphatic I, L, V	65	26	105	27	201	26
Aromatics F, W, Y	16	6	26	7	50	7
Positive K, R, H	39	16	42	11	77	10
Negative D, E	40	16	45	11	91	12
Tingy G, A, S	38	15	74	19	148	19
Total	249	100	395	100	761	100

RpaA role in the biological clock

The phosphorylation rhythm of the cyanobacterial circadian clock must be communicated to the rest of the cell to take advantage of this powerful device. Overwhelming evidence point to RpaA as the main conduit responsible for this propagation of timing information.[34,88,94,95] In 2013, RpaA became recognized as a global transcription regulator, rhythmically binding to more than 110 genome locations, including *kaiBC* gene controlling co-expression of KaiC and KaiB, thus the core oscillator is regulated by a transcription-translational feedback loop.[34] O'Shea generated a slew of complex global circadian phenotypes by controlling the phosphorylation of a single transcription factor, RpaA. When phosphorylated, RpaA is responsible for the activation of circadian dusk-specific gene expression that includes sigma factors and secondary effectors that cause a cascade of downstream expression of genes.[34] The O'Shea group also found that phosphorylation of RpaA was regulated by two-component antagonist histidine kinases, SasA and CikA. They demonstrated that SasA acts mostly as a kinase to phosphorylates and activates RpaA, while CikA predominately functions as a phosphatase to dephosphorylate and inactivates RpaA.[58] Further evidence to support the importance of SasA, CikA, and RpaA interactions was found by the Golden group utilizing statistical analysis of covariant residues among related amino acid sequences revealing a high degree of interaction between these three proteins.[96]

The structure of RpaA has only been predicted with homology-base approximations, however conserved OmpR/PhoP-like receiver (REC) domain and helix-turn-helix (HTH) DNA binding domain make ortholog structures likely accurate representations, shown in Figure 1.4. The phosphorylation site for RpaA is found within the response regulator/REC domain at residue D53 and has been mutated to create constitutively active and inactive phosphomimetics with D53E and D53A substitutions, respectively.[58]

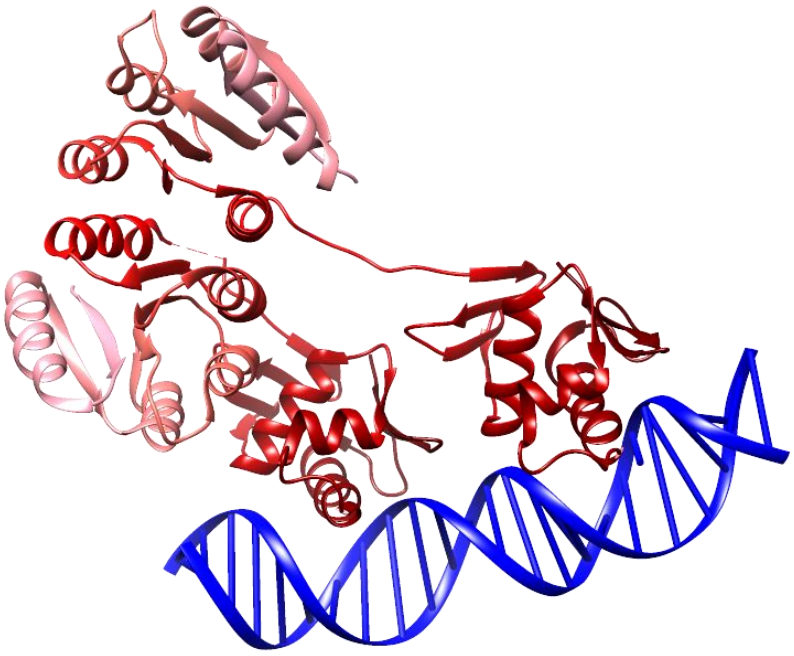

Figure 1.4. Crystal structure of dimeric RpaA ortholog in complex with promoter DNA. UCSF Chimera depiction of X-ray crystallographic structure of (red) hyperactive mutant transcriptional regulatory protein KdpE, sharing OmpR/PhoB-type domain with RpaA, in complex with (blue) promoter DNA.

Transcriptomic studies of *Synechococcus elongatus* homolog *Synechocystis elongatus*, RpaA, suggests that during the day it is mainly involved in the regulation of genes related to CO_2 accumulation while transitions to regulation of genes important for carbon metabolism at night.[97] This is consistent with *in vivo* studies highlighting RpaA as a necessary switch required for anticipatory accumulation of carbon reserves during the day, and then carbon catabolism at dusk.[98] This resource utilization and storage switching capability in cyanobacteria provided by RpaA allows for greater success during periods of prolonged darkness or limited resources.

SasA role in the biological clock

The primary known function of sensor histidine kinase, SasA, is transducing clock signaling from the core oscillator to the cyanobacterial cell. Namely, it is part of a two-component antagonistic histidine kinase pair, SasA and CikA, that translate the KaiC phosphorylation cycle into up-regulation or down-regulation of

gene expression through the transcription factor and master gene regulator, RpaA, respectively.[34,58,86–88] Although the molecular structure of full-length homodimer *Synechococcus elongatus* SasA has not been determined, the amino acid sequence has a high degree of homology to bacterial sensory kinase (SK), which was how the *sasA* gene was originally named, Synechococcus adaptive-response SK A.[86] However, recent collaboration between the LiWang and Partch labs elucidated an X-ray crystal structure of the N-terminal clock associating domain of SasA in complex with a truncated KaiC CI domain, PDB:6X61. This structure confirms previous homology predictions that the N-terminal domain of SasA adopts a thioredoxin-like fold, similar to that of fsKaiB, and competes with KaiB for the same binding interface on KaiC.[81,84,89]

CikA role in the biological clock

As previously stated here, CikA (Circadian Input Kinase A) is part of a two-component antagonistic histidine kinase in which it acts as a phosphatase towards the master gene regulator, RpaA. CikA is the largest of the three output pathway proteins and has a native homodimer oligomerization state and domains consistent with typical histidine protein kinases.[99] Originally, transposon mutagenesis was employed to identify genes linked to input pathways that aid in clock synchronization to external stimuli, and identified mutants without CikA expression were hindered.[87] There are four distinct domains including a N-terminal disordered region that has been linked to autophosphorylation activity, a GAF motif, a histidine protein kinase (HPK) with phosphorylation site H393, and a C-terminal receiver domain that interacts with core clock KaiBC complex.[56,87,99,100] Using *in vitro* trans-phosphorelay experiments, the Golden lab found that the C-terminal receiver domain of CikA lacks an invariable aspartyl residue that is phosphorylated by a cognate HPK resulting in an inability to accept a phosphoryl group from the kinase domain and was therefore given the classification as a pseudo-receiver (PsR) domain.[100] Interestingly, KaiA also has a pseudo-receiver domain and competes with CikA for the same binding site interface on the KaiBC nighttime complex.[56] Additionally, Golden and company found that KaiA, KaiC, and CikA colocalize to the poles of bacterium at night *in vivo*.[101,102] There is evidence suggesting CikA compensates for low KaiA concentration *in vitro* period effects, suggesting their competitive binding relationship infers cyanobacterial clock robustness.[103]

Output proteins SasA, CikA, and RpaA signal propagation

The extended oscillator of the cyanobacterial clock includes SasA, CikA, and RpaA and is very poorly understood at the mechanistic level. As stated previously, the general output pathway has been shown to function with SasA and CikA two-component antagonist regulated the activity of master gene regulator and transcription factor RpaA.[34,58,88] KaiC:SasA complex formation initiates autophosphorylation at residue H161 within SasA kinase domain, the phosphate group is then transferred to RpaA at residue D53, activating it for subsequent DNA binding to more than 110 genome locations.[34] Although, CikA was found to have much lower kinase activity than SasA, they both share highly similar histidine kinase domains and likely interact with RpaA with a similar interface. Orthologs of SasA or CikA and RpaA have been crystalized together providing insight into a predicted binding interface for phosphoryl transfer (Figure 1.5).[104,105]

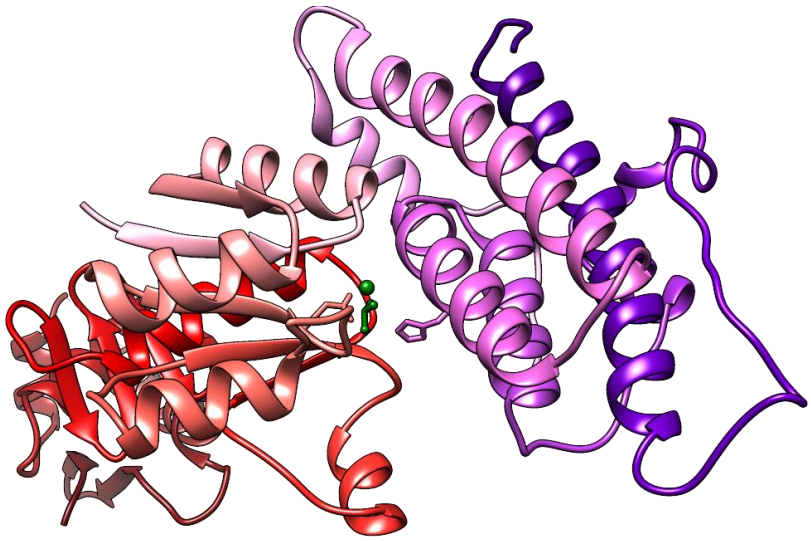

Figure 1.5. Structural orthologs for RpaA-SasA/CikA phosphoryl transfer interface, using OmpR/PhoB response regulator YPD1 and histidine kinase SLN1. UCSF Chimera depiction of two crystal structures PDB:2R25 showing YPD1 in complex with SLN1 representative of (red) RpaA and (purple) SasA/CikA, respectively, and PDB:1KGS crystal structure of OmpR/PhoB homolog from *Thermotoga maritima* which is similar to RpaA and superimposed here to extend the length of (red) SLN1 ortholog structure. Mg^{2+} and BeF_3^- ions are shown in green between representative residues for (purple) SasA-H161 or CikA-H393 and (red) RpaA-D53.

The temporal windows in which core clock timing information is transduced to the cyanobacterial cell through RpaA activity is restricted by Kai protein complex formations, shown in Figure 1.6. Namely, SasA has been shown to preferentially bind KaiC phosphomimetic representing pSpT and pST states.[81] Similar to SasA, KaiB binds the same phosphorforms of KaiC, with both proteins competing for the B-loop disordered region on KaiC CI domain, regulating the ability for SasA to be autophosphorylated and subsequent phosphoryl transfer to RpaA. Conversely, CikA can only interact with the core oscillator proteins when KaiB-KaiC nighttime complex is already formed. Upon ternary CikA-KaiB-KaiC complex formation, CikA phosphatase activity towards RpaA is initiated, thereby closing the short temporal window of SasA:RpaA downstream gene upregulation.

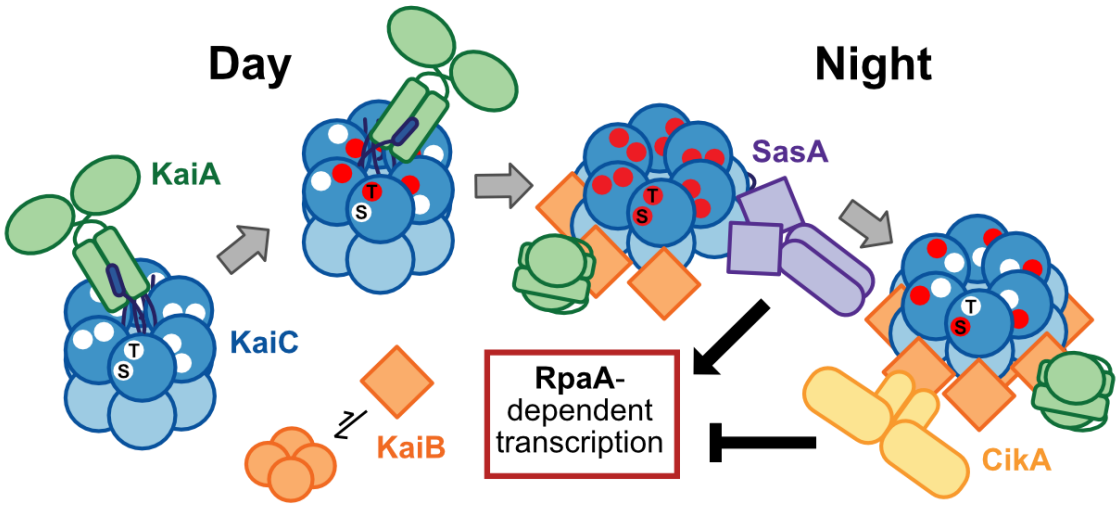

Figure 1.6. Cyanobacterial extended oscillator daytime and nighttime complex formations/dissociations. Same cartoon representation as in Fig. 1.2 with the addition of SasA and CikA output proteins and their downstream regulation of transcription factor, RpaA. SasA competes with KaiB for B-loop binding to promote kinase activity while CikA competes with KaiA to bind the KaiBC complex to initiate phosphatase activity on RpaA.

Chapter II

Development of fluorescence method to achieve the first real-time monitoring of a reconstituted circadian clock in a test tube

Technical development

Fluorescence techniques have contributed to significant advances in a wide range of biochemistry-related fields over the past 70+ years owing to a capacity for quantitative and precise measurements of dynamic reactions.[106] Perhaps the most famous example of improvements in fluorescence applications, the Nobel Prize in Chemistry to Osamu Shimomura, Martin Chalfie, and Roger Tsien for their discovery and development of GFP (green fluorescent protein).[107] Following this work, innumerable fluorescent probes have been added to the aspiring researcher's toolbox, making the selection of dyes with specific excitation and emission spectra an almost trivial step in method development. Parallel improvements in instrumentation, detectors, and computation power have further contributed to the explosion of fluorescence applications ranging from the co-expression of fluorescent proteins relating to transcription/translation in living organisms to measuring modulations in diffusion of single fluorescently labeled molecules *in vitro*.[108,109]

Dynamic protein-protein interactions can be quantitatively determined by monitoring change in fluorescence polarization of chemically labeled proteins of interest. There is a direct relationship between the rate of stochastic tumbling of molecules and their relative molecular size. Fluorescence anisotropy measurements involve photoselection of a fluorophore in a specified orientation and subsequent emission after the lifetime of the fluorophore, recording the change in polarization angle.[110] The randomization process is slower with larger molecules and excited-state lifetimes of many commercially available fluorophores are similar to the rotational correlation times of proteins (nanoseconds), giving fluorescence anisotropy measurements the ability to quantitatively measure the kinetics of protein-binding events (Figure 2.1).

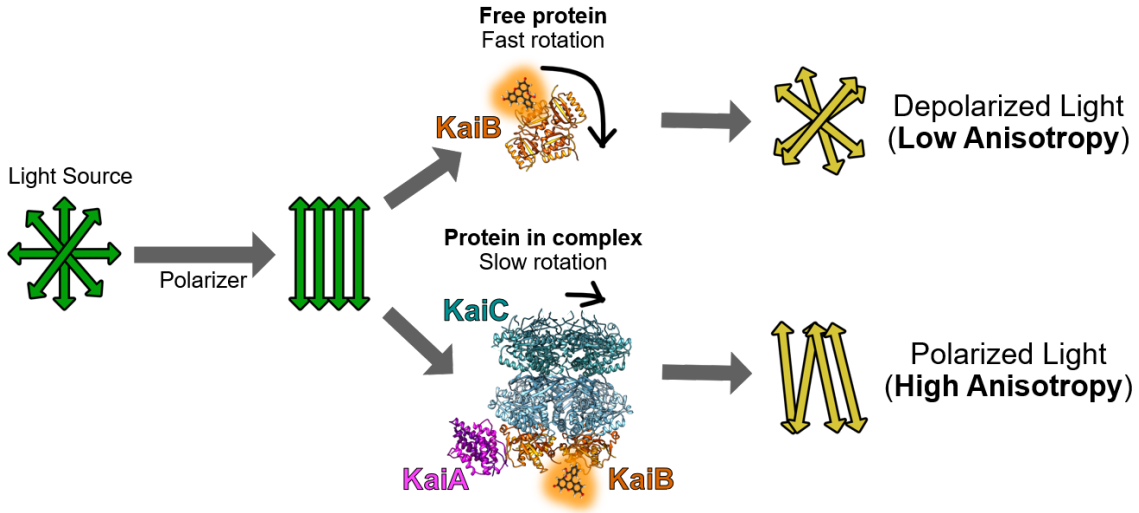

Figure 2.1. Cartoon of how labeled-KaiB protein free and ternary KaiA-KaiB-KaiC complexes have significantly different fluorescence anisotropies. The difference in tumbling rates of free KaiB and ternary KaiABC complex result in conservation of polarized light emission through the lifetime of the fluorophore (~4 ns) with the (bottom) complex, while free (top) free KaiB tumbling rate results in depolarization of light emitted from the conjugated fluorophore. Rotational correlation times of the fluorophore can be extrapolated following a calibration of fluorescence anisotropy with neat fluorescein in the presence of glycerol, which has defined values.[111,112]

An important step in preparation for anisotropy measurements is proper conjugation of a fluorophore to protein(s) of interest. A common method to accomplish this is thiol-mediated conjugation in which a maleimide or iodo group linked to a fluorophore is covalently bound to a cysteine residue -SH group on the protein of interest (Figure 2.2).[113–115] Modification of a protein amino acid sequence, through DNA mutagenesis, or addition of a cysteine to the N- or C-terminus may all be employed depending on the specific protein, its' function, and the molecular interaction being observed. Fluorescent labeling is not trivial and often requires labeling proteins of interest in multiple ways before determining the optimal conjugation site.

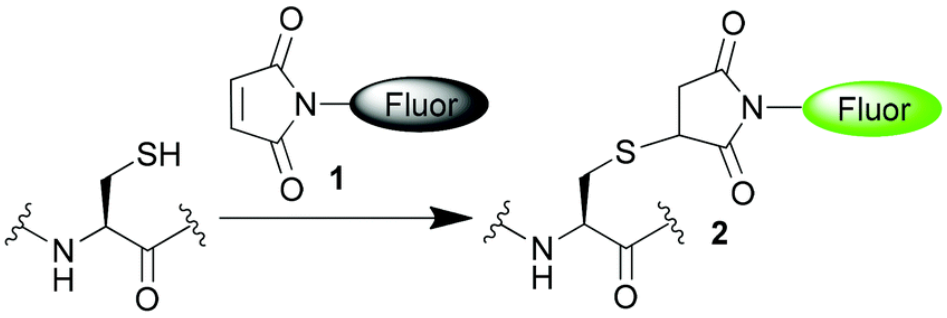

Figure 2.2. Depiction of how thiol-mediated fluorophore conjugation takes place. Simplified cysteine sidechain (-SH group) shown before and after fluorophore attachment.[116] This reaction is performed at 7.0 – 7.5 pH to ensure amines are protonated, preventing off target labeling.

Labeling of cyanobacterial clock proteins

Identifying the best candidate for a fluorescently labeled probe within the circadian clock system requires multiple considerations. First, fluorescence polarization measurements allow for differentiation in molecule size which in this context would be protein-protein complex formation and dissociation.[106,115] Within the KaiABC oscillator rhythmic complex formation is known to occur with KaiA interacting with KaiC during the subjective day and KaiA, KaiB, and KaiC forming a ternary complex at subjective night.[56] Because both KaiA and KaiC are thought to be in complex with one another throughout the day, it suggests that monitoring a change in molecular size of these proteins may not result in meaningful fluorescence polarization differences. Second, the method of fluorophore conjugation presented here is thiol-mediated and therefore proteins lacking native cysteines are preferred. This is often advantageous over amine-reactive reagents due to less than half the number of cysteines residues found in mammalian proteins than lysine residues.[117] Standard labeling approaches utilize a 7.0 to 7.5 pH solution to avoid iodoacetamide reacting with amines by ensuring their protonated.[118] The goal is fluorophore attachment to any solvent exposed cysteine residue. The most common way to rationally label a protein in a specific location is to substitute all unwanted cysteine labeling sites to an alanine or serine. Alanine is most common substitution because it scores high in the BLOcks SUbstitution Matrices or BLOSUM, however serine is sometimes used due to its close relationship with cysteine structurally in addition to a moderate predicted BLOSUM score (Figure 2.3).[119] Making amino acid substitutions will always risk functional changes in proteins of interest, and therefore minimizing these modifications is ideal. Comparing amino acid sequences of the Kai proteins

shows that KaiB has no native cysteines whereas both KaiA and KaiC have many (see Chapter I).

BLOSUM 62 scoring matrix

(positive values are shaded)

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	**4**																			
R	-1	**5**																		
N	-2	0	**6**																	
D	-2	-2	**1**	**6**																
C	0	-3	-3	-3	**9**															
Q	-1	**1**	0	0	-3	**5**														
E	-1	0	0	**2**	-4	**2**	**5**													
G	0	-2	0	-1	-3	-2	-2	**6**												
H	-2	0	**1**	-1	-3	0	0	-2	**8**											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	**4**										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	**2**	**4**									
K	-1	**2**	0	-1	-3	**1**	**1**	-2	-1	-3	-2	**5**								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	**1**	**2**	-1	**5**							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	**6**						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	**7**					
S	**1**	-1	**1**	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	**4**				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	**1**	**5**			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	**1**	-4	-3	-2	**11**		
Y	-2	-2	-2	-3	-2	-1	-2	-3	**2**	-1	-1	-2	-1	**3**	-3	-2	-2	**2**	**7**	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	**3**	**1**	-2	**1**	-1	-2	-2	0	-3	-1	**4**

Figure 2.3. BLOSUM62 matrix. Positive values imply the substitution is more likely than a random amino acid substitution, while negative values represent a substitution that is less likely than random. BLOSUM62 means the matrix was built from aligned sequences with no more than 62 % sequence identity.[119]

A fluorescently labeled KaiB protein would yield large modulations in molecular size through daytime inactivity and nighttime complex formation and does not contain native cysteine residues that would need to be substituted. However, choosing KaiB as a candidate for fluorophore attachment requires an additional consideration because it undergoes fold-switching from a ground state tetramer to a rare fold-switched monomer (Figure 2.4A).[84] Critically, the first half of KaiB tertiary structure remains the same in both conformations while the secondary structures in the second half of KaiB swap from alpha helices to beta strands and vice versa, resulting in the active monomeric fold-switched state that is stabilized in a nighttime complex with KaiC.[56,84] The intramolecular tetramer and dimer interfaces must be conserved to avoid disrupting metamorphic activity. The final consideration for fluorophore attachment is protein-protein binding interfaces. KaiB is known to bind KaiC and KaiA to form the nighttime recessive complex in addition to interacting with output histidine kinase CikA.[56,84] This oligomeric and

conformational metamorphism, along with known intermolecular binding interfaces with KaiC, KaiA, and CikA were considered when selecting Lys25 for mutation to cysteine and thiol-mediated fluorophore conjugation (Figure 2.4). Standard molecular cloning, protein expression, and labeling procedures were used (Appendix B). Site-directed DNA mutagenesis with Quickchange PCR,[120] allowed for a single-step polymerase chain reaction protocol to achieve Lys25 to Ala25 substitution on a plasmid vector containing a kaiB insert [112].

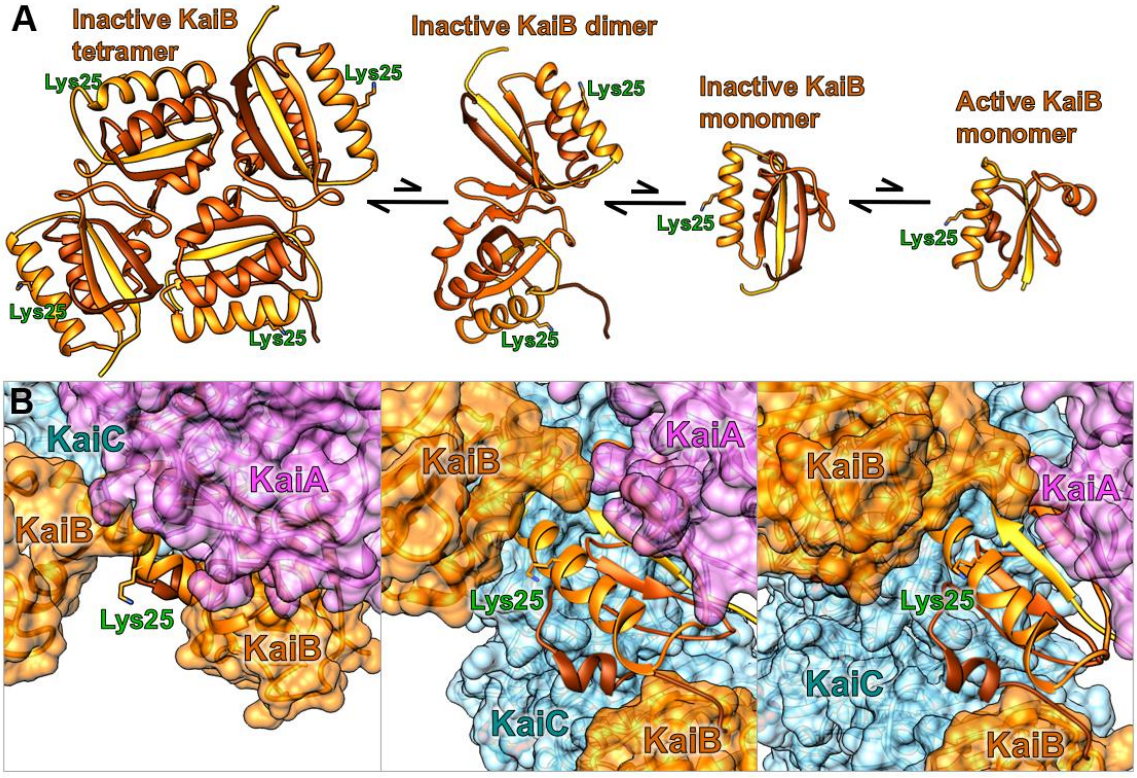

Figure 2.4. Fluorescence labeling at lysyl 25 of KaiB. A) KaiB's four different free states and (B) ternary KaiABC complex (PDB: 5JWQ) shown in multiple orientations, with (purple) KaiA, (orange) KaiB, and (blue) KaiC.[112]

Verification of fluorescently labeled KaiB-K25C construct functionality was achieved by reconstituting the Kai oscillator - 1.2 µM KaiA, 3.5µM KaiC, and 3.5 µM total KaiB with 0.05 µM labeled KaiB probe - *in vitro* and monitoring fluorescence anisotropy for more than ten days (Figure 2.5). This method and mutant KaiB construct were shared with collaborators prior to publishing and utilized in several primary research efforts, allowing for direct observations of real-time population shifts between daytime free KaiB and bound KaiB at night.[112,121–123] The fluorescence anisotropy rhythm of labeled KaiB has been

shown to lag behind KaiC phosphorylation rhythm by nearly 4 hours, shown by orthogonal gel electrophoresis densitometry phosphorylation assay first developed by Kondo and coworkers (Figure 2.7).[31] Here, the fluorescence method presented provides higher temporal resolution (minutes vs. hours), does not require aliquots of the reconstituted oscillator reaction to be removed which both ensures no perturbations and removes the time window limitations that come with losing volume at each time point, can be run in a multi well plate reader making it substantially higher throughput, directly informs on protein-protein interactions as opposed to indirect inferences made from KaiC phosphorylation gel electrophoresis assays, and can be employed to monitor any biological clock component from core KaiABC to output SasA, CikA, RpaA, and DNA binding rhythms.

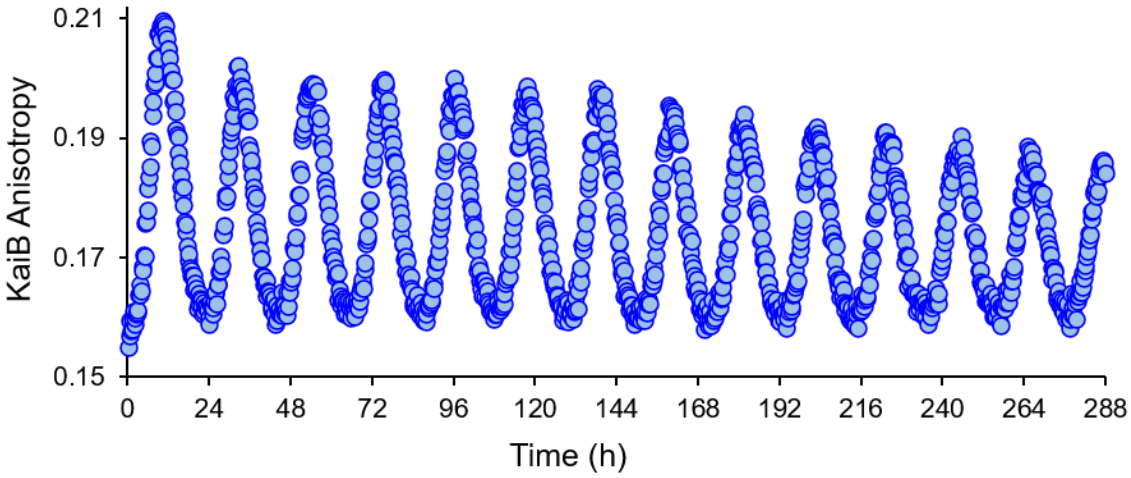

Figure 2.5. *In vitro* reconstituted oscillator at standard 3.5 μM KaiC, 3.5 μM KaiB, and 1.2 μM KaiA concentrations. Fluorescently labeled KaiB probe, at 0.05 μM concentration, plotted against incubation time.

Clock system questions to be answered

The *in vitro* cyanobacterial clock system has been a hotbed of profound discoveries over the course of the last decade. Within that time some key advances include; limits of KaiA and KaiB concentrations have been established,[124] day/night transitions in ATP/ADP ratios were linked to modulations in KaiC phosphorylation,[38] the CI and CII domains of KaiC stack rhythmically to drive the oscillator,[47] output proteins SasA and CikA pathway for gene expression through master regulator RpaA,[34,58,89] KaiB/SasA competition is

a key mechanism of output,[81] mixtures of oscillators with different phases synchronize through negative feedback,[93] KaiB ring formation is cooperative,[83] KaiC CI ATPase activity contributes to clock slowness,[73,74] KaiB fold-switching activity discovered,[84] core oscillator complexes determined,[56] visualization of KaiA-KaiC change in affinity relative to phosphor-state,[125] real-time observation of cyanobacterial protein-protein interactions,[112] and CikA has been shown to compensate for low concentrations of KaiA.[103] Despite these advances the near 24-h biological rhythm of the cyanobacterial circadian oscillator and output signal propagation mechanisms remain mysterious. Specifically, why is the *in vitro* oscillator less resilient to change in core component concentrations than *in vivo* and what methodologies in the future may aid in providing answers in addition to elucidating new insightful questions?

Tracking the ordered temporal KaiC pattern of phosphorylation - S/T $\rightarrow$ S/pT $\rightarrow$ pS/pT $\rightarrow$ pS/T $\rightarrow$ S/T $\rightarrow$ …, where S and T represent residues Ser431 and Thr432, the two phosphorylation sites of KaiC, and pS and pT denote their phosphorylated states[68,69] has allowed for numerous insights into the cyanobacterial clock, such as KaiA stimulates KaiC autophosphorylation during the day, and KaiB inhibits KaiA in order to promote KaiC autodephosphorylation at night.[39,46,50,67,78,124] However, the real-time phase relationships of these events are not known. The flexibility of the fluorescence methodology developed here presents an avenue to uncover these temporal relationships (Figure 2.6).

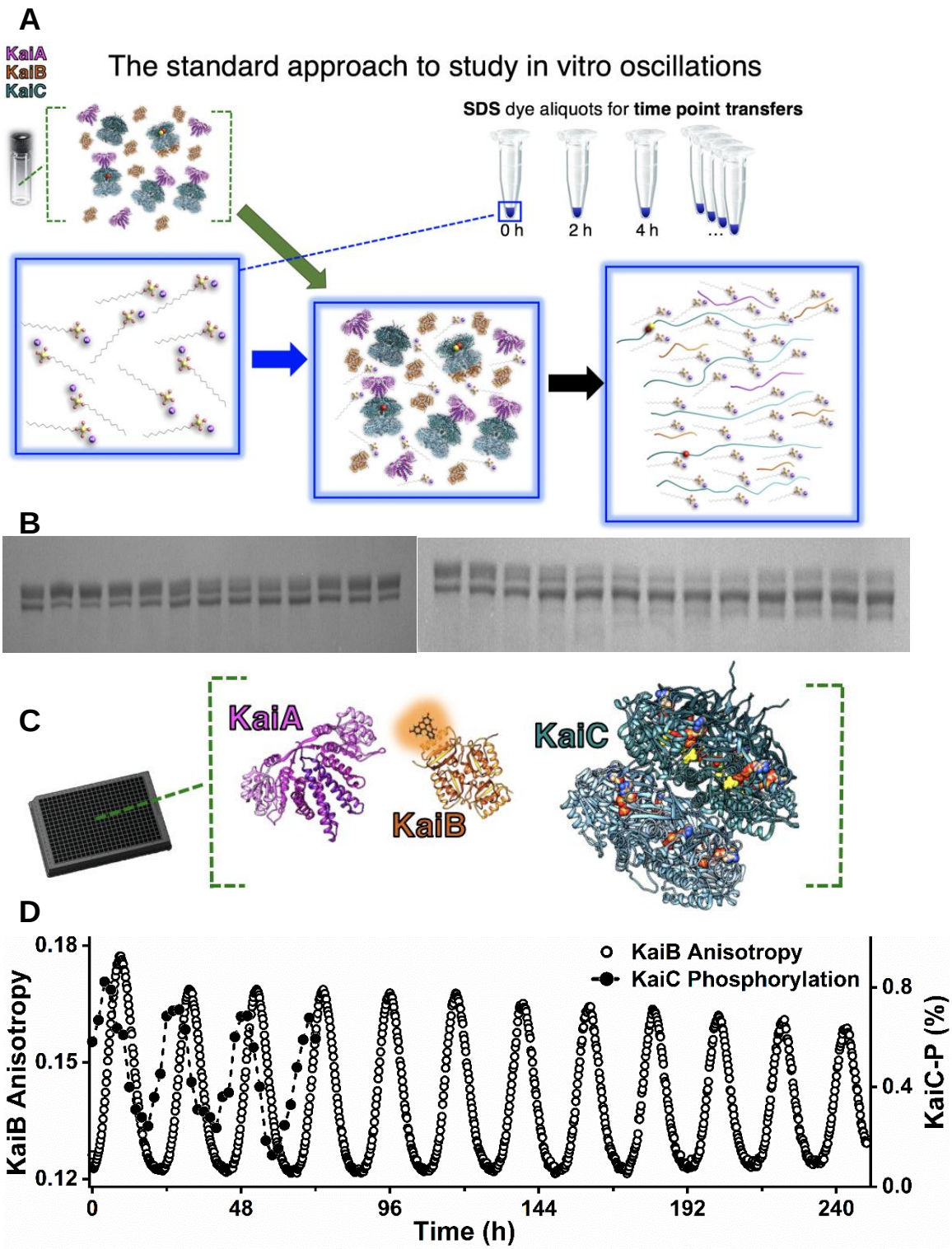

Figure 2.6. Difference in assay procedure for (left) KaiC phosphorylation assay and (right) fluorescence binding assay. A and B) Visual representation of the KaiC phosphorylation assay aliquot collection and (B) example of SDS-PAGE showing change in KaiC phosphoryl states over time. C) Visual representation of novel FP-PTO methodology and high-throughput multi-well

plate capability. D) KaiC phosphorylation rhythm and FP-PTO KaiB anisotropy rhythm both plotted against time.

Advantages of high throughput monitoring of *in vitro* oscillations under many reaction conditions

Utilizing fluorescence polarization anisotropy of a labeled clock proteins not only improves flexibility but allows for high-throughput, drastically increased experimental data collection capability, and higher temporal resolution allows for improved quantitative analysis of period and phase determinations under different conditions (Figure 2.6D). Previously, Kondo and coworkers explored how changes in KaiA and KaiB concentrations adversely effected the core KaiABC clock.[124] There findings provided a window of KaiA and KaiB concentrations that produced KaiC phosphorylation rhythms. However, employing fluorescently labeled KaiB probe fluorescence polarization of post translational oscillator (FP-PTO) methodology provided higher sensitivity, broadening the observable limit for decreased levels of KaiA and KaiB, shown in Figure 2.7. Critically, this analysis showed that the core oscillator KaiA concentration dependence is independent of KaiB levels above 1.4 µM, in which all reactions with 0.3 µM KaiA above that threshold of KaiB displayed nearly identical KaiB anisotropy rhythms. Monitoring fluorescently labeled KaiB provides further insight as well, such that the first peak of 0.3 µM KaiA conditions is uniformly larger than subsequent daily peaks. This suggests that the initial KaiC phosphorylation state prior to synchronization was much higher than was reached thereafter. This can be attributed to the low level of KaiA present not being sufficient to instigate KaiC autophosphorylation due to a relatively high concentration of KaiB inhibiting KaiA activity.[39,46,50,67,78,124]

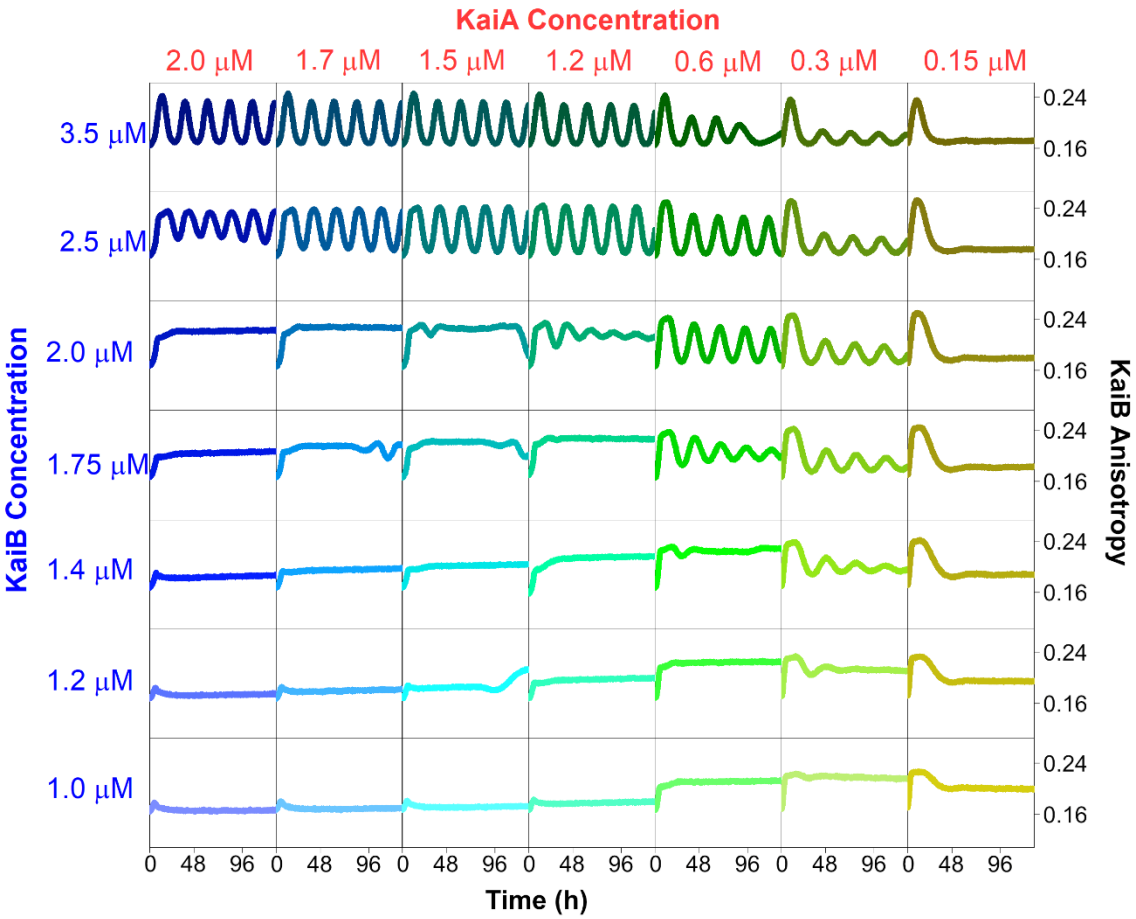

Figure 2.7. FP-PTO raw KaiB anisotropy rhythms over 120 hours under different KaiA and KaiB concentration ratios. Oscillator conditions are performed with fixed KaiC 3.5 μM and varied (red, top) KaiA and (blue, left) KaiB concentrations.

To better understand the initial KaiC phosphorylation conditions for the reactions shown in Figure 2.7, SDS-PAGE analysis was performed on freshly prepared KaiC proteins (Figure 2.8). It is known that KaiC autodephosphorylates when incubated at 30°C and autophosphorylated when incubated on ice,[69] however detailed monitoring of these reactions has not been observed. Here, the rate of KaiC autophosphorylation on ice is ~ 2% per hour with linear regression fit (R^2 = 0.9861) of the first 12 hours on ice with fully dephosphorylated KaiC starting conditions (Figure 2.8). Also, of note is the high 74% KaiC-P freshly prepare protein from E. coli mediated expression (see Appendix A). This initial KaiC phosphorylation condition is the reason for a larger first, synchronization, KaiB fluorescence anisotropy peak observed under limiting KaiA concentrations, shown in Figure 2.7.

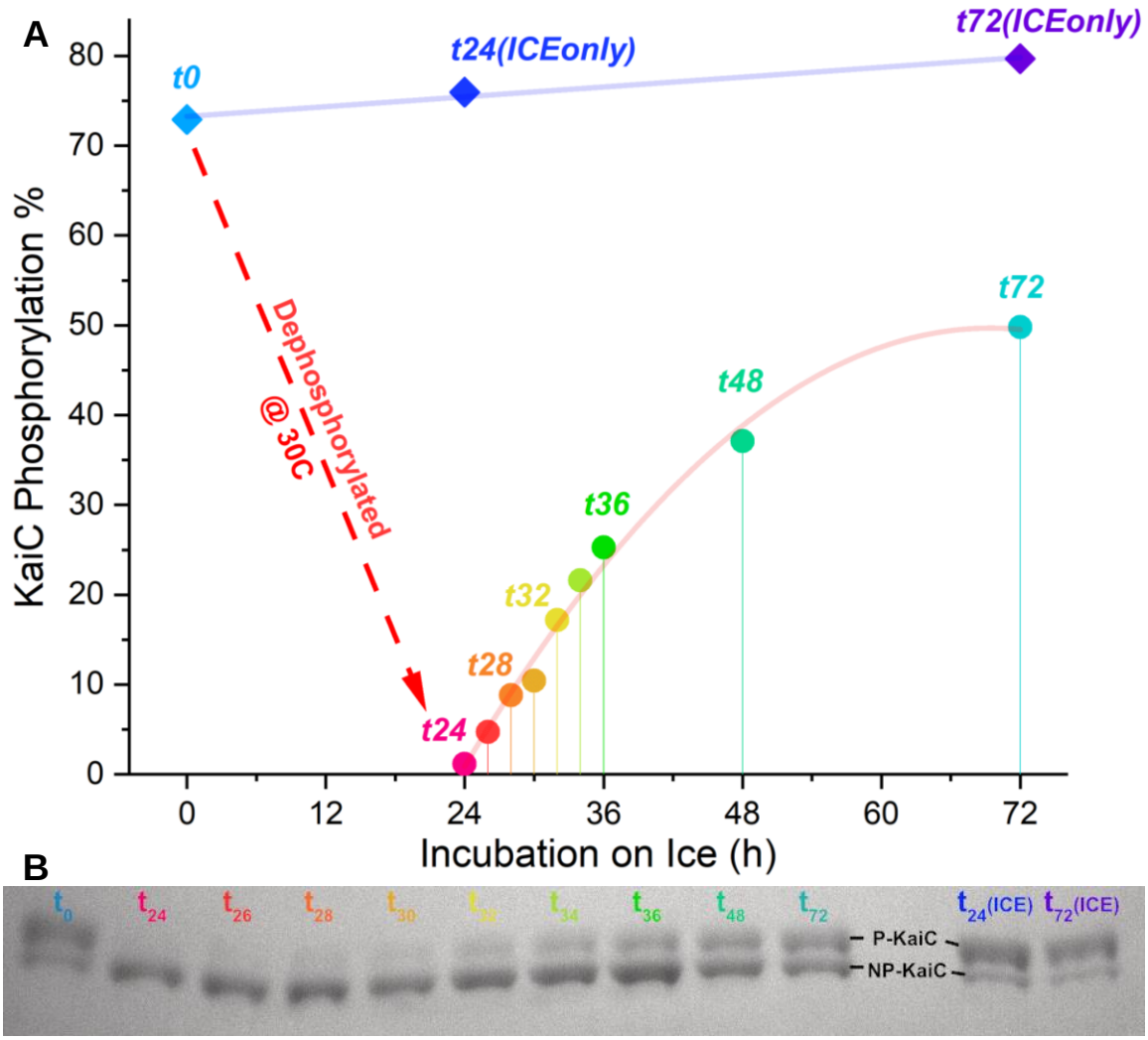

Figure 2.8. KaiC autophosphorylation on ice calibration curve. A) KaiC phosphorylation assay monitoring KaiC autophosphorylation on ice of two samples that were either incubated on ice directly after protein expression or incubated at 30°C for 24 hours then placed on ice. B) Photograph of SDS-PAGE highlighting phosphorylated (P-KaiC) and nonphosphorylated (NP-KaiC) bands for aliquots taken from (blue to purple) sample incubated on ice following expression or (rainbow) sample incubated on ice following full autodephosphorylation of KaiC by 30°C water bath for 24 hours.

Observing fluorescence anisotropy of a labeled KaiB probe within a free running *in vitro* KaiABC reaction, in near real-time, has the added benefit of directly showing how an oscillator begins and ultimately fails. In Figure 2.7, reactions containing limiting concentrations of KaiB (conditions that did not produce binding rhythms) and 1x KaiA (1.2 μM) or higher, displayed an initial spike in KaiB

anisotropy and then plateaued to some steady-state equilibrium. Critically, the equilibrium level is dependent on the relative ratio of KaiA, such that lower concentrations of KaiA result in more KaiB bound at the first peak and/or after oscillator desynchrony (Figure 2.9). These in vitro oscillations capture in real-time how KaiA and KaiB regulate one another's complex formation with KaiC, consistent with previous KaiC phosphomimetic studies with KaiA and KaiB.[81,93,126] If there is enough KaiB present, during the initial synchronization peak, KaiB forms adequate KaiB-KaiC binary complexes that sequester and recruit the population of KaiA, forming KaiA-KaiB-KaiC ternary nighttime complex which halts further KaiC phosphorylation completing the negative feedback loop necessary for binding rhythms.[55,56]

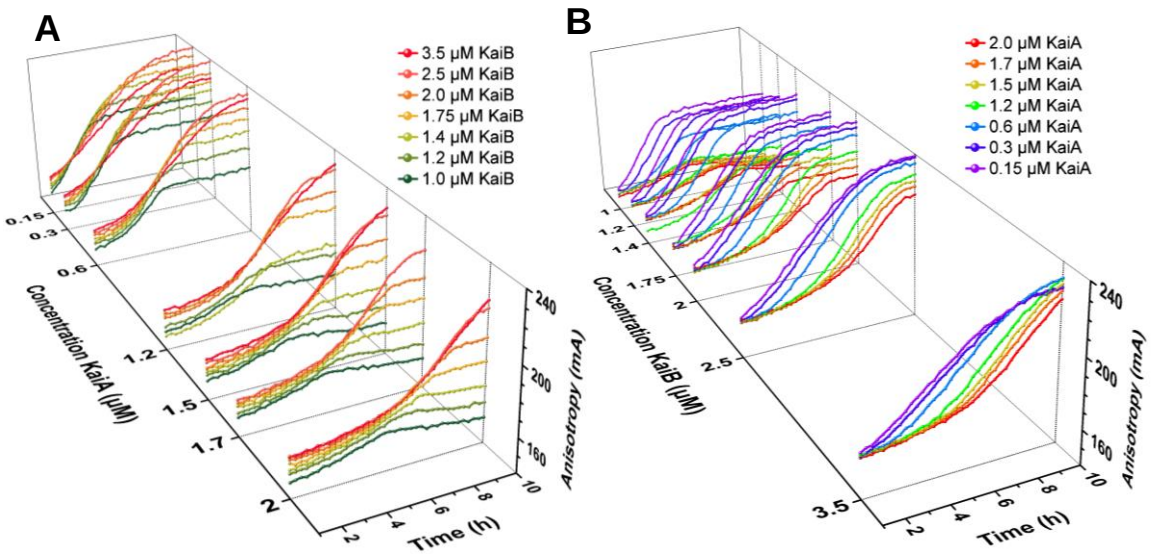

Figure 2.9. First 10 hours of KaiABC *in vitro* FP-PTO reactions with varied KaiA and KaiB concentrations. A) KaiB anisotropy values plotted with changes in concentration of KaiA on the z-axis with KaiB concentrations represented by (green to red) from low to high, respectively. B) KaiB anisotropy values plotted with changes in concentration of KaiB on the z-axis with KaiA concentrations represented by (purple to red) from low to high, respectively. Both (A and B) are showing the same representative data from two independent experiments.

Phase mixing of *in vitro* KaiABC oscillator reactions

The cyanobacterial clock phase response to external stimuli has long been an area of interest among chronobiologists. Groundbreaking work by the O'Shea lab showed that modulating ATP/ADP ratios in KaiABC oscillators resulted in tunable

phase shifts *in vitro*.[38] Namely, the addition of ADP resulted in shifts to KaiC autodephosphorylation phase, suggesting that a decrease in illumination can entrain the clock to nighttime activity. Initial efforts to observe oscillators in different phases being mixed was performed with KaiC phosphorylation assay tracking changes in densitometry of phosphorylated (P-KaiC) and nonphosphorylated (NP-KaiC) bands with gel electrophoresis, finding that mixtures of KaiC phosphorylation populations default to dephosphorylation if either of the parent oscillators are in that phase.[127] However, insights beyond initial change in phase were not reported due to the laborious technique limiting the time duration these experiments were monitored. Here, recapitulating phase mixing experiments with high throughput fluorescently labeled KaiB polarization monitoring allows for substantially improved qualitative and quantitative analysis (Figure 2.10). Upon mixing Kai reactions of any phase, there is a decrease in period that overtime, on the timescale of days, that tracks back towards the period length of parent oscillations. Notably, phases six to nine hours offset of one another when combined result in lower initial period lengths (3h + 9h, 3h + 12h, 6h + 12h). The daughter oscillations show a faster period increase over time following mixing, suggesting the core cyanobacterial *in vitro* clock system has a stable limit cycle around 21-h, but the pull to that period length is only gradual. This observation was only possible due to the drastically increased experimental monitoring and high temporal resolution obtained with the FP-PTO methodology. Future work elucidating mechanisms responsible for this short period synchronization effect will likely be facilitated by this approach.

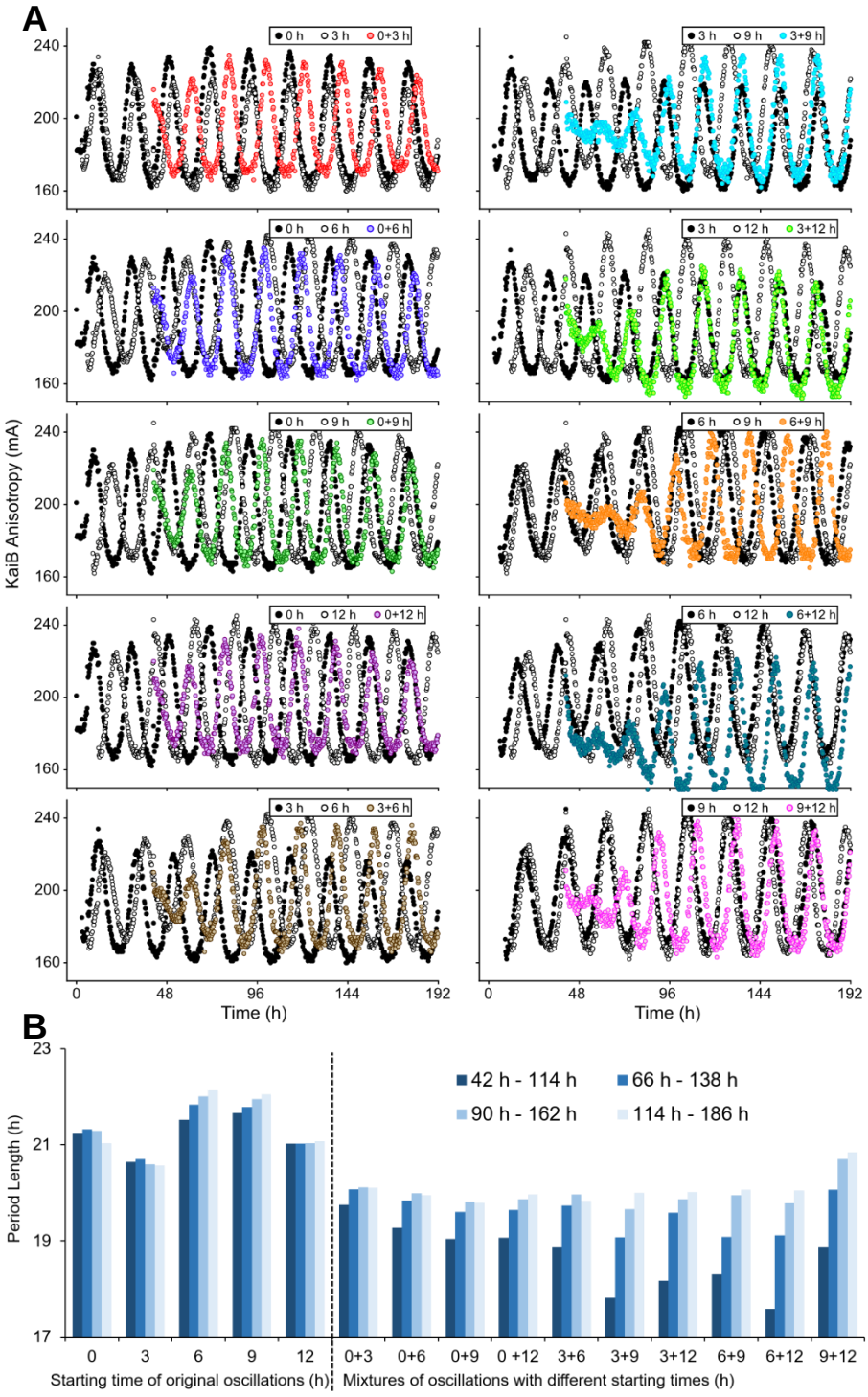

Figure 2.10. *In vitro* Kai oscillator reaction mixtures of different phases. A) Oscillations containing 1.2 μM KaiA, 3.45 μM KaiB, 3.5 μM KaiC, and 50 nM fluorescently labeled KaiB probe run at 30°C were started at time 0 h, 3 h, 6 h, 9 h, and 12 h and were mixed at 42 h in all possible combinations. Mixed phase oscillations are plotted as fluorescence anisotropy of probe KaiB against

incubation time 0-192 hours, with (black closed and open circles) original reactions and (colored) mixtures. B) Period determinations calculated using BioDare2 online period analysis suite[128,129] fitting scrolling time windows (dark to light).

Adding output components SasA, CikA, and RpaA to create the first extended oscillator *in vitro*, monitored in real time

Given the added flexibility of the fluorescence method over that of the standard KaiC phosphorylation assay, the next step was to fluorescently label additional cyanobacterial clock components. As mentioned previously, thiol-mediated fluorophore attachment relies on conjugation to existing native cysteine -SH groups, or directed mutagenesis to achieve specific attachment locations. Due to the large number of native cysteines present in KaiA, KaiC, SasA, CikA, and RpaA, multistep PCR was utilized (Appendix C). The structure of full length *Synechococcus elongatus* SasA, CikA, and RpaA proteins have not be documented in the literature. However, each of these output proteins can be easily predicted based on shared homology via SWISS-Model online suite,[130] and are shown in Figure 2.11. All three proteins are most likely homodimer oligomers, with some evidence to support SasA may trimerize[89] which has more recently been contradicted.[85]

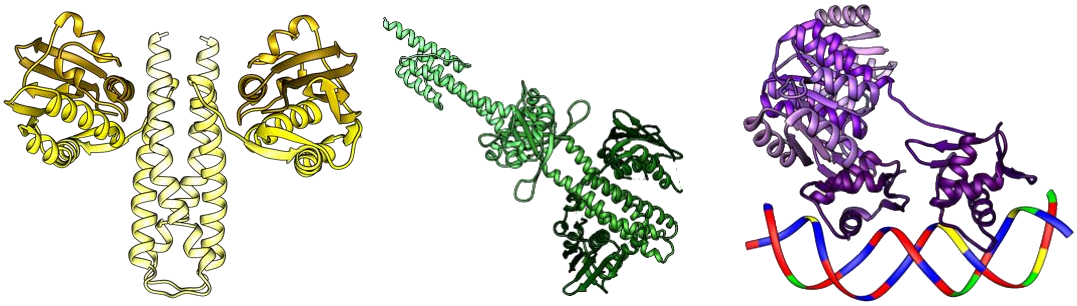

Figure 2.11. Homology based structures of CikA, SasA, and RpaA binding to promoter *kaiBC* DNA sequence. UCSF Chimera ribbon representations of (yellow) CikA, (green) SasA, and (purple) RpaA bound to (rainbow) nucleotide DNA oligo.

Although full length structures of SasA and CikA have not been published, the truncated monomeric pseudo receiver domain of CikA ($CikA_{psr}$) has been

resolved previously by NMR and the crystal structure of N-terminal thioredoxin domain of SasA ($SasA_{trx}$) in complex with CI domain of KaiC is shown in Chapter III, Fig. 3.1, 3.3.[56] The known structures of $CikA_{psr}$ and $SasA_{trx}$, and their corresponding binding interfaces with KaiB-KaiC complex and CI domain of KaiC, respectively, these truncated domains for the first step towards fluorescently labeling the full extended oscillator *in vitro* (Figure 2.12).

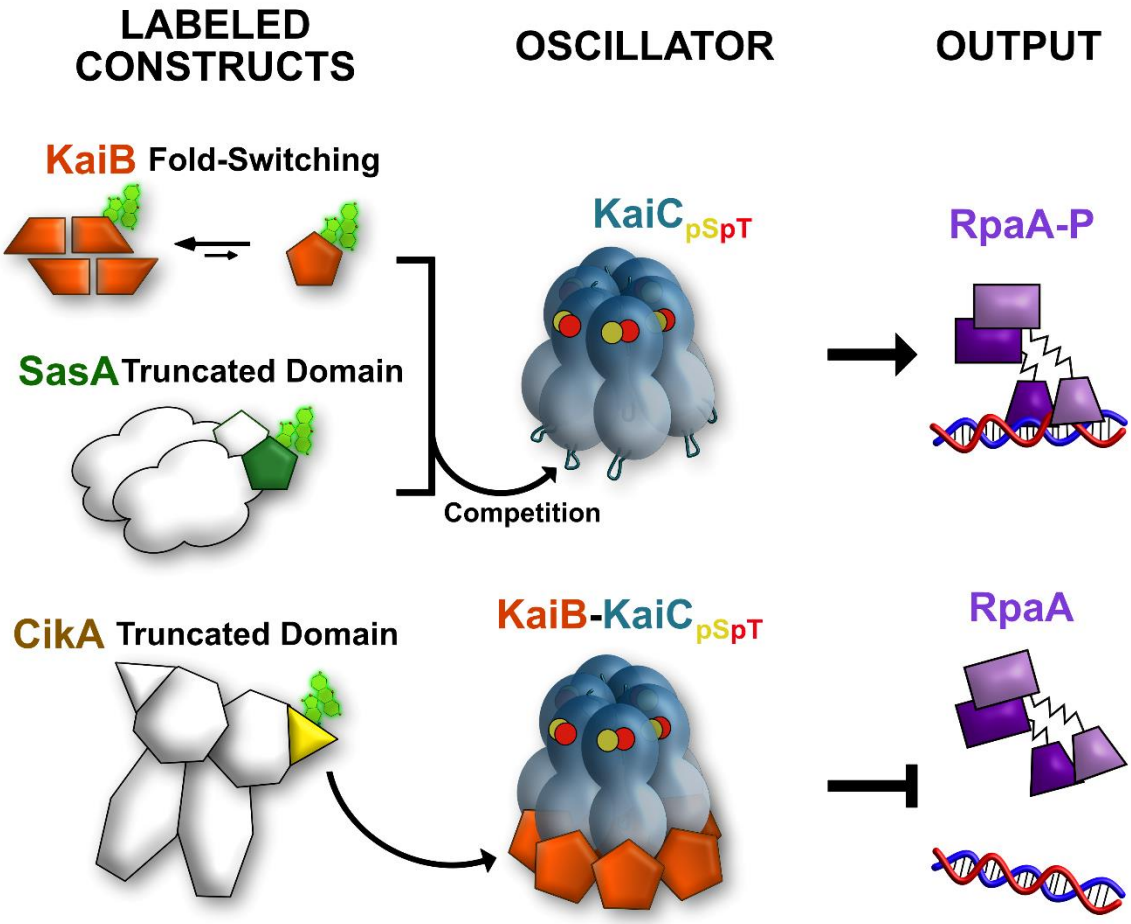

Figure 2.12. KaiC complex formations and downstream signal propagation that can be captured with real-time fluorescence spectroscopy. Left side, diagram showing cartoon representing fluorescent labeling sites on KaiB (orange), SasA truncated N-terminal domain (dark green), and CikA truncated PsR domain (yellow) with fluorophore (light green). Center, highlights where different fluorescently labeled constructs interact with KaiC core oscillator component. Right, depicts bound-state downstream action on master regulator RpaA (purple).

Successful fluorophore conjugation of $SasA_{trx}$ and $CikA_{psr}$ domains allowed for quantitative phase determinations of both output kinases relative to KaiB anisotropy and KaiC phosphorylation, presented in Chapter III, excluding $CikA_{psr}$ which is shown in Figure 2.13 compared to KaiB anisotropy. As expected, the CikA domain is in phase with KaiB because it interacts with the Kai proteins only when KaiB is in the nighttime recessive complex with KaiC.[56] A list of mutant KaiA, KaiB, KaiC, SasA, CikA, and RpaA constructs that were produced for this specific aim are presented in Table 2.1, representing various truncations, cysteine substitutions, and stability tags. The purpose of these mutant constructs was to establish the first full extended oscillator capable of tracking clock timing and signal propagation from core to output, culminating in rhythmic binding of transcription factor RpaA to *kaiBC* promoter DNA, with every component including DNA fluorescently labeled and monitored in near real time.

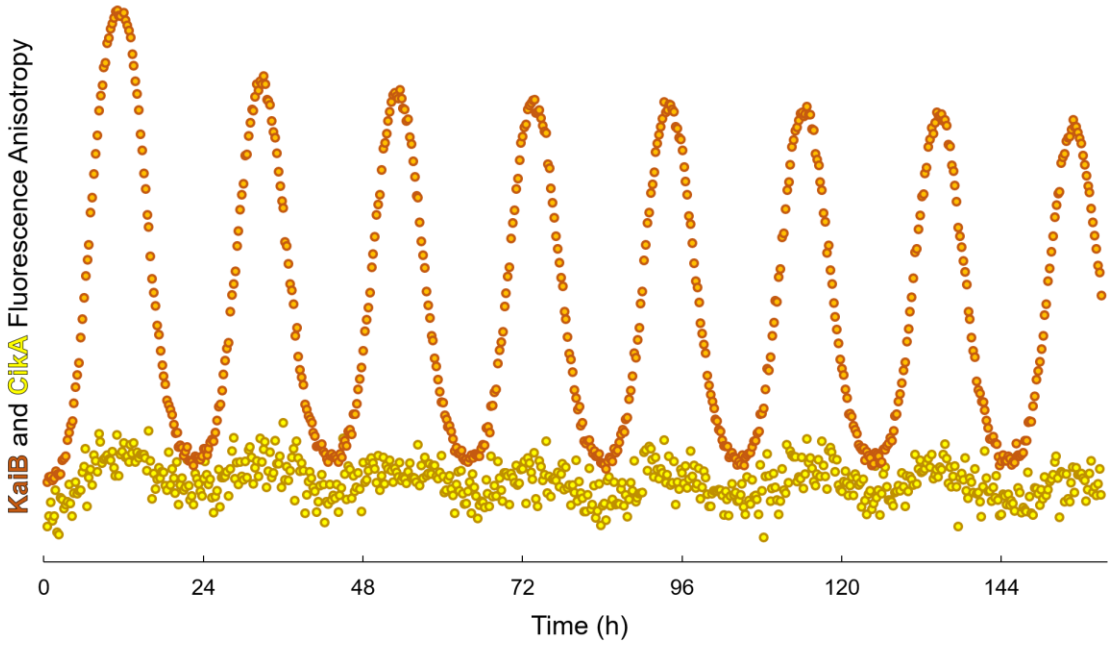

Figure 2.13. Fluorescently labeled CikApsr and KaiB in KaiABC oscillator monitored in real time. Fluorescence anisotropy of (orange) KaiB and (yellow) $CikA_{psr}$ are plotted against incubation time in 1x oscillator conditions at 30°C.

Table 2.1. Mutant constructs prepared for fluorescence labeling of the cyanobacterial core and extended oscillator.

KaiB	**KaiC**
SeKaiB	FLAG-SeKaiC-16-247-R40A-K172A-FLAG
SeKaiB-FLAG	FLAG-SeKaiC-16-247-R40A-K172A-FLAG-E128C
SeKaiB-FLAG-K25C	FLAG-SeKaiC-16-247-R40A-K172A-FLAG-E128C-6IAF
SeKaiB-FLAG-K25C-6IAF	FLAG-SeKaiC-C274A-C306A-C348A
SeKaiB-FLAG-K25C-Alexa488	FLAG-SeKaiC-C274A-C306A-C348A-E128C
SeKaiB-FLAG-K25C-Alexa594	FLAG-SeKaiC-C274A-C306A-C348A-Cys
SeKaiB-FLAG-K25C-EM5	FLAG-SeKaiC-ET
SeKaiB-FLAG-N29C	FLAG-ThKaiC-17-247-R41A-K173A-FLAG
SeKaiB-FLAG-N29C-6IAF	
SeKaiB-FLAG-N29C-EM5	
KaiA	**RpaA**
SeKaiA	SeRpaA
SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-FLAG-K246C	SeRpaA-C65A-C169A-C229A
SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-FLAG-K246C-6IAF	SeRpaA-C65A-C169A-C229A-E16C
SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-FLAG-K246C-Alexa488	SeRpaA-C65A-C169A-C229A-I36C
SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-FLAG-K246C-EM5	SeRpaA-C65A-C169A-C229A-T63C
SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-FLAG-R249C	SeRpaA-C65A-C169A-C229A-S12C
	SeRpaA-C65A-C169A-C229A-S12C-Alexa488
	SeRpaA-D53A
	SeRpaA-D53E
SasA	**CikA**
FLAG-SeSasA	FLAG-SeCikA
FLAG-SeSasA-1-225-C196A-FLAG-Q31C	SeCikA
FLAG-SeSasA-1-225-C196A-FLAG-Q31C-Alexa488	SeCikA-S605-606-754
FLAG-SeSasA-FLAG	

FLAG-SeSasA-P13A-13-103-FLAG	SeCikA-S605-606-754-C644S-C686S-C742S
FLAG-SeSasA-P13A-13-103-FLAG-Q31C	SeCikA-S605-606-754-C644S-C686S-C742S-S727C
FLAG-SeSasA-P13A-13-103-FLAG-Q31C-6IAF	SeCikA-S605-606-754-C644S-C686S-C742S-S727C-6IAF
FLAG-SeSasA-P13A-13-103-FLAG-Q31C-Alexa488	SeCikA-S605-606-754-C644S-C686S-C742S-S727C-EM5
FLAG-SeSasA-P13A-13-103-FLAG-Q31C-Alexa594	SeCikA-S605-606-754-C644S-C686S-C742S-T736C
FLAG-SeSasA-P13A-13-103-FLAG-Q31C-EM5	SeCikA-S605-606-754-C644S-C686S-C742S-T736C-Alexa488
FLAG-ThSasA-P16-16-107-FLAG	SeCikA-S605-606-754-C644S-C686S-C742S-T736C-Alexa594
SeSasA-FLAG	FLAG-SeCikA-377-754-C401S-C644S-C686S-C742S-T736C
SUMO-FLAG-SeSasA-FLAG	FLAG-SeCikA-377-754-C401S-C644S-C686S-C742S-T736C
SUMO-SeSasA-FLAG	

Fluorescently labeled DNA to monitor gene expression *in vitro*

In addition to the monitoring of protein-protein interactions, utilizing fluorescence labeling can easily be extended to nucleotides. RpaA is a transcription factor that is activated by histidine kinase SasA and deactivated by phosphatase CikA, and is responsible for the regulation of more than 100 rhythmically expressed genes in cyanobacteria.[34] The *kaiBC* promoter sequence that RpaA binds[58] was used here to show how fluorescently labeled RpaA can report protein-protein and protein-nucleotide interactions (Figure 2.14). This fluorescently labeled full length RpaA construct is shown to not interact with the nucleotide *kaiBC* promoter sequence of DNA until phosphomimetic KaiC-ET (S431E), SasA, and DNA are present together, at which point the fluorescence anisotropy increases suggesting binding. Sequentially adding fold-switched KaiB (fsKaiB) does not readily perturb RpaA anisotropy, but addition of CikA produces another increase in RpaA anisotropy. The equilibrium anisotropy value of RpaA grows in presence of CikA resulting from ternary CikA-KaiB-KaiC complex formation activating CikA phosphatase activity and thereby CikA association with RpaA. Ultimately, this experiment verifies the fluorescently labeled RpaA construct is functional and can report SasA and CikA associations. Additional controls are necessary to confirm

that this mutations and fluorophore attachment are not disrupting RpaA-DNA complex formations.

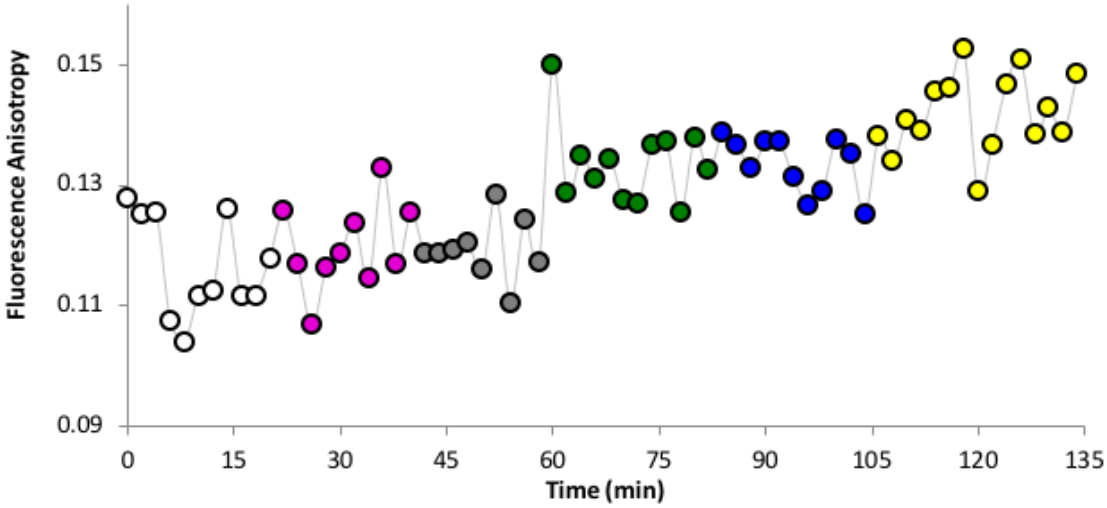

Figure 2.14. RpaA functional test in one cuvette. Free fluorescently-labeled 0.05 µM RpaA-Alexa488 (white) has multiple components added step-wise at the start of each color transition, starting with 0.5 µM DNA promoter sequence that RpaA has been found to associate with (pink), 1 µM KaiC-ET phosphomimic (grey), 0.32 µM SasA (green), 1 µM fsKaiB (blue), and 0.32 µM CikA (yellow).

Chapter III

Structural mimicry confers robustness in the cyanobacterial circadian clock

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*These authors contributed equally to this work

Abstract

The histidine kinase SasA enhances robustness of circadian rhythms in the cyanobacterium *S. elongatus* by temporally controlling expression of the core clock components, *kaiB* and *kaiC*. Here we show that SasA also engages directly with KaiB and KaiC proteins to regulate the period and enhance robustness of the reconstituted circadian oscillator *in vitro*, particularly under limiting concentrations of KaiB. In contrast to its role regulating gene expression, oscillator function does not require SasA kinase activity; rather, SasA uses structural mimicry to cooperatively recruit the rare, fold-switched conformation of KaiB to the KaiC hexamer to form the nighttime repressive complex. Cooperativity gives way to competition with increasing concentrations of SasA to define a dynamic window by which SasA directly modulates clock robustness.

One Sentence Summary

SasA controls the assembly of clock protein complexes through a balance of cooperative and competitive interactions.

Submitted

Conceptualization, J.H., J.A.S., C.L.P., and A.L.; Methodology, J.H., J.A.S., and C.R.B.; Investigation, J.H., J.A.S., J.G.P., C.S., D.C.E., R.K.S., and S.T.; Validation, J.H., J.A.S., and S.T.; Formal Analysis, J.H., J.A.S., C.R.B., and P.C.; Resources, S.S.G; Data Curation, J.H. and J.A.S.; Writing – Original Draft, J.H., J.A.S., and C.L.P.; Writing – Reviewing and Editing, J.H., J.A.S., D.C.E., C.R.B., S.S.G., C.L.P., and A.L.; Funding Acquisition, J.H., J.P., P.C., S.S.G., C.L.P., and A.L.; Supervision, S.S.G., C.L.P., and A.L.

Main Text

The core circadian clock genes *kaiA, kaiB, and kaiC*, are essential for rhythmic gene expression in cyanobacteria[23] and their proteins generate a ~24-hour rhythm of phosphorylation of KaiC *in vivo*[52] that persists in the absence of transcription or translation.[32] Reconstitution of this post-translational oscillator (PTO) *in vitro*[31] has led to deep insight into the structural and kinetic mechanisms that regulate formation of the clock protein assemblies and underlie cyanobacterial circadian rhythms. KaiC autophosphorylation is stimulated by KaiA during the day,[46,53,55,72] whereas autodephosphorylation is favored at night when KaiB binds to KaiC and sequesters KaiA (**Fig. 3.1A**).[55,56] Although these three proteins alone can establish a circadian oscillation *in vitro*, two histidine protein kinases contribute to the complex *in vivo*: SasA and CikA, which coordinate a vast program of circadian gene expression executed by the response regulator, RpaA.[34] The N-terminal thioredoxin-like domain of SasA is structurally homologous to a rare fold-switched monomer state of KaiB, and directly competes with this form of KaiB for binding to KaiC.[81,84] This structural similarity and binding competition raised the possibility that SasA helps to regulate formation of the nighttime repressive complex, which is known to be restricted by (*i*) the phosphorylation state of the KaiC CII domain,[93] (*ii*) the intrinsically slow rate of ATP hydrolysis by the KaiC CI domain,[73,131] and (*iii*) the rare conversion of KaiB to the its fold-switched form that is competent to bind KaiC.[84] Similarly, CikA and KaiA bind to the same site on the KaiBC complex,[56] and this competition shortens the period[84] and compensates for diminished concentrations of KaiA in the PTO *in vitro*,[103] suggesting that output kinases fortify robustness of the PTO by modulating Kai protein interactions directly.

To explore how the intrinsic structural similarity between fold-switched KaiB and the N-terminal thioredoxin-like domain of SasA might influence the cyanobacterial PTO, we took advantage of a new high-throughput fluorescence polarization-based post-translational oscillator (FP-PTO) assay that reports on complex formation with KaiC in real time.[112] This allowed us to capture circadian rhythms of quaternary complex formation with KaiC under standard assay conditions for the PTO[31] in the presence of 50 nM fluorescently-labeled probes for KaiB or the thioredoxin-like domain of SasA, $SasA_{trx}$, benchmarking the phase of these rhythms to the KaiC phosphorylation cycle (**Fig. 3.1B** and **3.2**).[69]

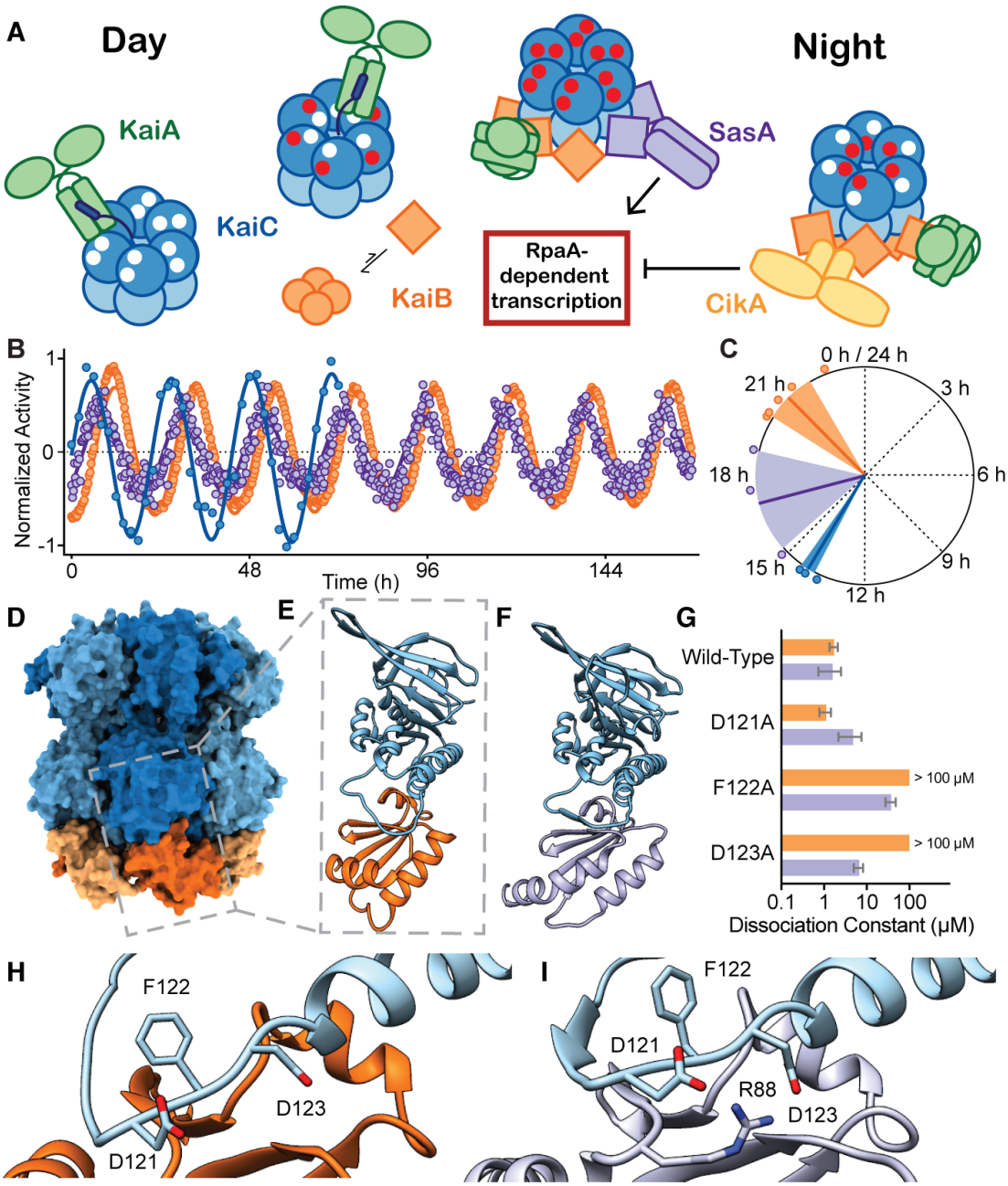

Figure 3.1. SasA and KaiB bind with sequential but overlapping phases to the same site on KaiC. A) Cartoon schematic of the cyanobacterial oscillator and associated output pathways. The KaiC phosphorylation rhythm[69] is depicted with white (unphosphorylated) or red (phosphorylated) circles. Ground-state KaiB tetramer (circles) interchanges with the fold-switched thioredoxin-like fold (squares). **B**) Normalized rhythms of KaiC phosphorylation (blue, measured by SDS-PAGE for 72 h) with KaiB or $SasA_{trx}$ association with KaiC (orange or purple, respectively). Data points from individual traces (n=3) are overlaid in circles, and the mean (solid line) fitted to a sine function. See **Fig. 3.2** for raw

data. **C**) Rayleigh phase diagram of KaiC phosphorylation and KaiB or $SasA_{trx}$ binding for *in vitro* oscillations. Dark line, mean phase of KaiC binding or phosphorylation (circles represent n ≥ 3 assays; SD, width of the light wedge). Calculated phases are KaiB: 20.9 ± 0.9 h, SasA: 17.1 ± 1.7 h, and KaiC-P: 14.0 ± 0.4 h. **D**) The KaiB-KaiC hexamer (PDB:5JWQ). Subunits of KaiC are depicted in alternating light/dark blue, and fsKaiB monomers alternate yellow/orange. Gray box, position of the KaiC-CI domain bound to KaiB. **E**) The KaiC-CI domain-fsKaiB subcomplex (PDB:5JWO), with KaiC in blue, KaiB in orange. **F**) The KaiC-CI domain-$SasA_{trx}$ subcomplex (PDB:6X61), with KaiC in blue, KaiB in orange. **G**) Equilibrium dissociation constants (K_D) of KaiC-EE and mutants for KaiB or $SasA_{trx}$ (mean ± SEM, n = 3). Where indicated, binding was too weak for curve-fitting and K_D is reported at > 100 μM. **H-I**) The interface of KaiC-CI with fsKaiB (**H**) or $SasA_{trx}$ (**I**) with key residues highlighted.

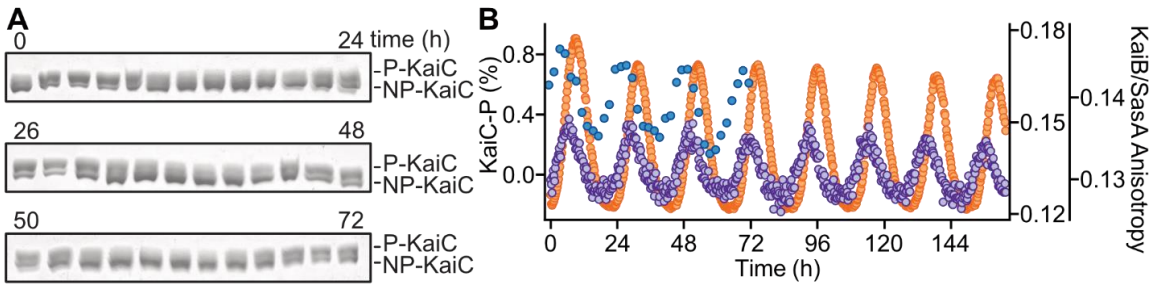

Figure 3.2. KaiC phosphorylation and FP-PTO of fluorescently-labeled KaiB and SasA. **A**) KaiC phosphorylation rhythms under the standard oscillator concentrations of 3.5 μM KaiC, 3.5 μM KaiB, and 1.2 μM KaiA *in vitro*. P-KaiC, phosphorylated KaiC; NP-KaiC, non-phosphorylated KaiC. **B**) Overlay of KaiC phosphorylation (KaiC-P) rhythm (blue) with unnormalized (raw) FP values for fluorescently-labeled KaiB probe (orange, inner right y-axis) or fluorescently-labeled $SasA_{trx}$ probe (purple, outer right y-axis) from distinct FP-PTO assays set up under standard *in vitro* oscillator conditions and plotted against the start of incubation, with time zero representing the moment KaiC was added to the reactions(see **Fig.** 3.**1B** for normalized data). The smaller change in anisotropy exhibited by the $SasA_{trx}$ probe may reflect more transient association with KaiC.

The phase of $SasA_{trx}$ binding preceded that of KaiB by a few hours and was closer to the peak of KaiC phosphorylation (**Fig. 3.1C**), similar to that of full-length SasA. Using KaiC phosphomimetics KaiC-EE and KaiC-EA, which are widely used to approximate the dusk-like pS,pT and nighttime-like pS,T states, we found that KaiB has similar affinity for both (**Fig. 3.3**). By contrast, full-length SasA has a higher preference for the earlier-occurring pS,pT state.[89,93] Although

the N-terminal domain of SasA is necessary and sufficient for binding to KaiC,[132] avidity effects in the full-length dimer enhanced affinity for KaiC-EE by at least two orders of magnitude compared to the isolated, monomeric $SasA_{trx}$ domain (**Fig. 3.3**).[85] One other contribution to the delayed phase of KaiB-KaiC binding could be its population shift from a highly stable tetrameric KaiB ground state to an unstable monomeric fold.[84]

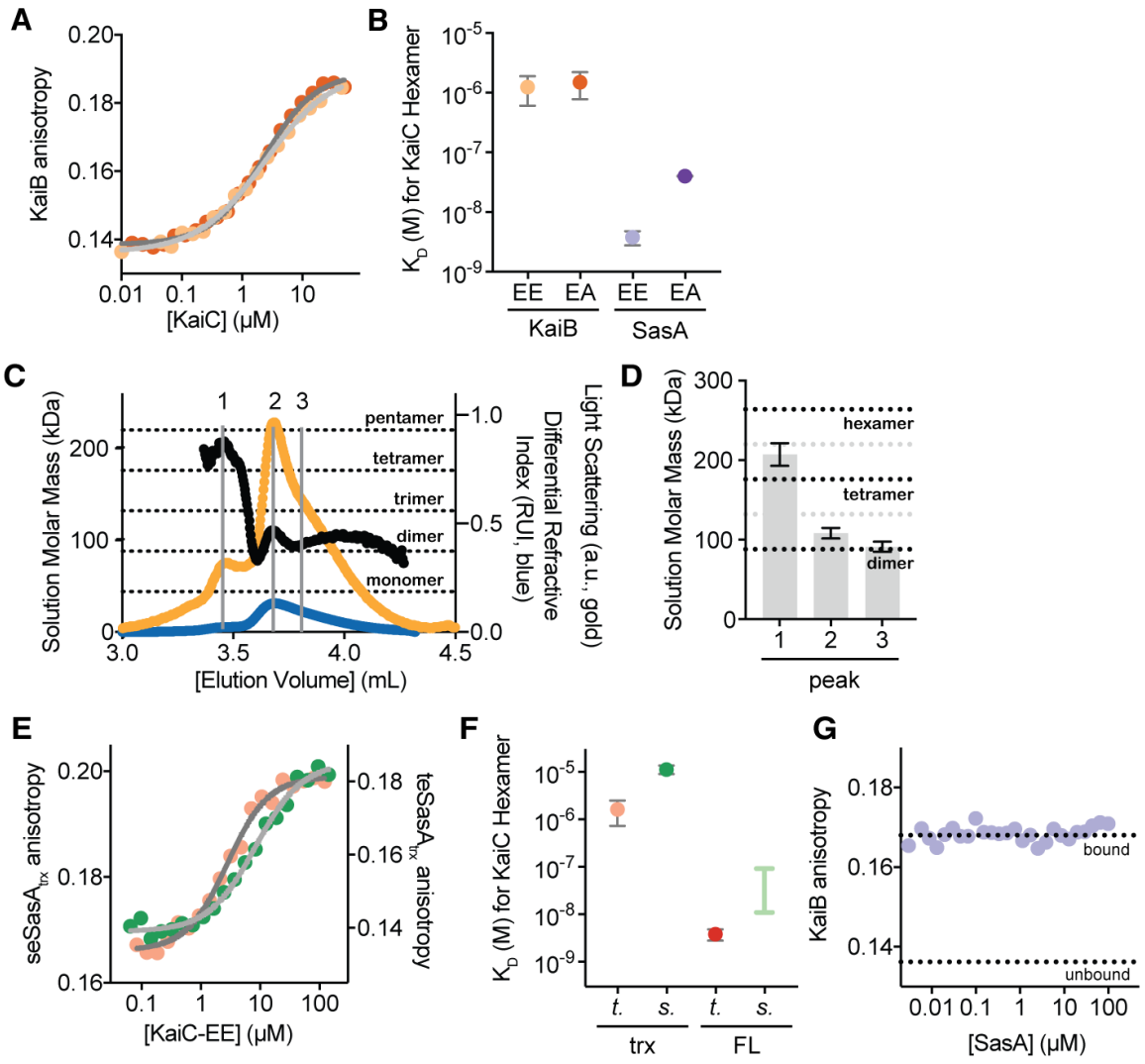

Figure 3.3. Biochemical properties of the SasA-KaiC interaction. A) KaiB binds to KaiC-EE and KaiC-EA with similar affinity. Representative equilibrium binding assays of 50 nM fluorescently-labeled KaiB from *S. elongatus* in the presence of increasing concentrations of *S. elongatus* KaiC-EA (dark orange) and KaiC-EE (light orange). Data were fit to a Langmuir binding isotherm (EA, dark gray; EE, light gray). **B**) SasA binds KaiC more tightly than does KaiB, with

a preference for the pS/pT phosphomimetic, KaiC-EE. Calculated equilibrium dissociation constants (K_D) from binding data in panel **A** (mean ± SD, n = 3; light and dark orange) are compared to affinities from Valencia et al.[89] (mean ± SD, K_D measured by SPR; light and dark purple) for full-length SasA from *T. elongatus*. **C**) Full-length SasA exists primarily a dimer in solution. Traces from a representative size-exclusion chromatography coupled to multiangle light scattering (SEC-MALS) run for full-length SasA from *S. elongatus*. Differential refractive index (blue) is depicted with light scattering (gold) and absolute mass estimation (kDa, black line). **D**) Quantitative mass analysis of peaks indicated at the top of panel **C** (peaks 1-3) analyzed from triplicate SEC-MALS runs are consistent with a dimer-tetramer equilibrium (mean mass in kDa ± SD). Calculated masses for dimer, tetramer and hexamer are represented by black dotted lines. **E**) The $SasA_{trx}$ domain of *T. elongatus* binds with higher affinity than the *S. elongatus* $SasA_{trx}$ domain to the respective KaiC-EE variant from each organism. Equilibrium binding assays of fluorescently-labeled $SasA_{trx}$ from *S. elongatus* (dark green) or *T.* (pink) *elongatus*. Data were fit as in panel **A**. **F**) Full-length SasA (FL) binds much more tightly than the isolated $SasA_{trx}$ domain (trx), suggesting that it relies on avidity for efficient binding to KaiC hexamer. Calculated K_D values extracted from data in panel **E** (mean ± SD, n = 3; pink or dark green) are compared to affinities from Valencia et al.[89] (mean ± SD, measured by SPR; red) for full-length SasA from *T. elongatus*. The affinity range of *S. elongatus* SasA calculated from the 2D titration data and our thermodynamic model (**Fig.** 3.**7F** and **3.5**) is represented as light green bars. **G**) Full-length SasA does not display avidity and compete KaiB off of the KaiC-C1 monomer. Fluorescently-labeled KaiB was bound to 10 μM monomeric KaiC-CI domain and subsequently titrated with full-length SasA as indicated. Anisotropy values for bound and unbound KaiB (dashed lines) were determined from curve fits in **Fig.** 3.**5A**.

Six monomers of the active, thioredoxin-like fold of KaiB assemble onto the KaiC hexamer of *S. elongatus*[55] and the related thermophilic species, *T. elongatus*[56] to nucleate formation of the nighttime repressive state (**Fig. 3.1D**). A high-resolution crystal structure of the sub-complex comprising a single KaiC-CI domain and a fold switch-locked mutant of KaiB I88A (I87A in *S. elongatus*, referred to herein as “fsKaiB” for both) illustrates how KaiB docks onto the exposed B-loop of the CI domain (**Fig. 3.1E**). We solved a crystal structure of the $SasA_{trx}$ domain bound to the KaiC-CI domain from *T. elongatus*, revealing that SasA binds the B-loop in a similar orientation to KaiB (**Fig. 3.1F** and **Table 3.4**). To interrogate the importance of this interface, we probed several KaiC residues in the B-loop by mutagenesis and found that substitutions at sites conserved between *S. elongatus* and *T. elongatus* (F122 and D123) decrease affinity for both KaiB and

SasA (**Fig. 3.1G-I** and **3.4**). We then examined how differences in the structures of the thioredoxin-like domains of KaiB and SasA, or their orientations on the B-loop might influence binding to adjacent subunits of the KaiC hexamer (**Fig. 3.4**). Modest changes in the length and orientation of the C-terminal helix between the SasA and KaiB thioredoxin-like folds could lead to steric clashes of SasA with a neighboring subunit. Consistent with this idea, saturation binding experiments showed that $SasA_{trx}$ domains cannot fully occupy all six binding sites on the KaiC hexamer (**Fig. 3.4**). Moreover, unlike the highly cooperative binding observed for KaiB, SasA does not associate cooperatively with KaiC.[83,85]

Table 3.1. Refinement statistics for structure determination of KaiC-CI-$SasA_{trx}$ complex.

Data collection	
Space group	$P2_1$
Cell dimensions	
a, *b*, *c* (Å)	107.6, 121.58, 133.59
α, β, γ (º)	90.0, 108.78, 90.0
Resolution (Å)	49.05-3.2 (3.30-3.20) *
No. of total reflections	153257 (13790)
No. Unique Reflections	53267 (4637)
R_{merge}	16.5 (75.7)
R_{pim}	13.0 (57.5)
I/σI	5.5 (1.5)
Completeness (%)	98.8 (99.3)
$CC_{1/2}$	0.95 (0.58)
Wilson B-factor	58.3
Redundancy	2.9 (3.0)
Refinement	
Resolution (Å)	3.20
R_{work}/ R_{free}	22.2/26.6
No. atoms	
Protein	15469
Ligand/ion	30
B-factors	
Protein	61.3
Ligand/ion	46.1
R.m.s deviations	
Bond lengths (Å)	0.003
Bond angles (º)	0.78
Residues in favored	93.1
Residues in outlier	0.6

*Highest resolution shell is shown in parenthesis. One crystal was used for data collection. Data were collected at a wavelength of 1.0 Å.

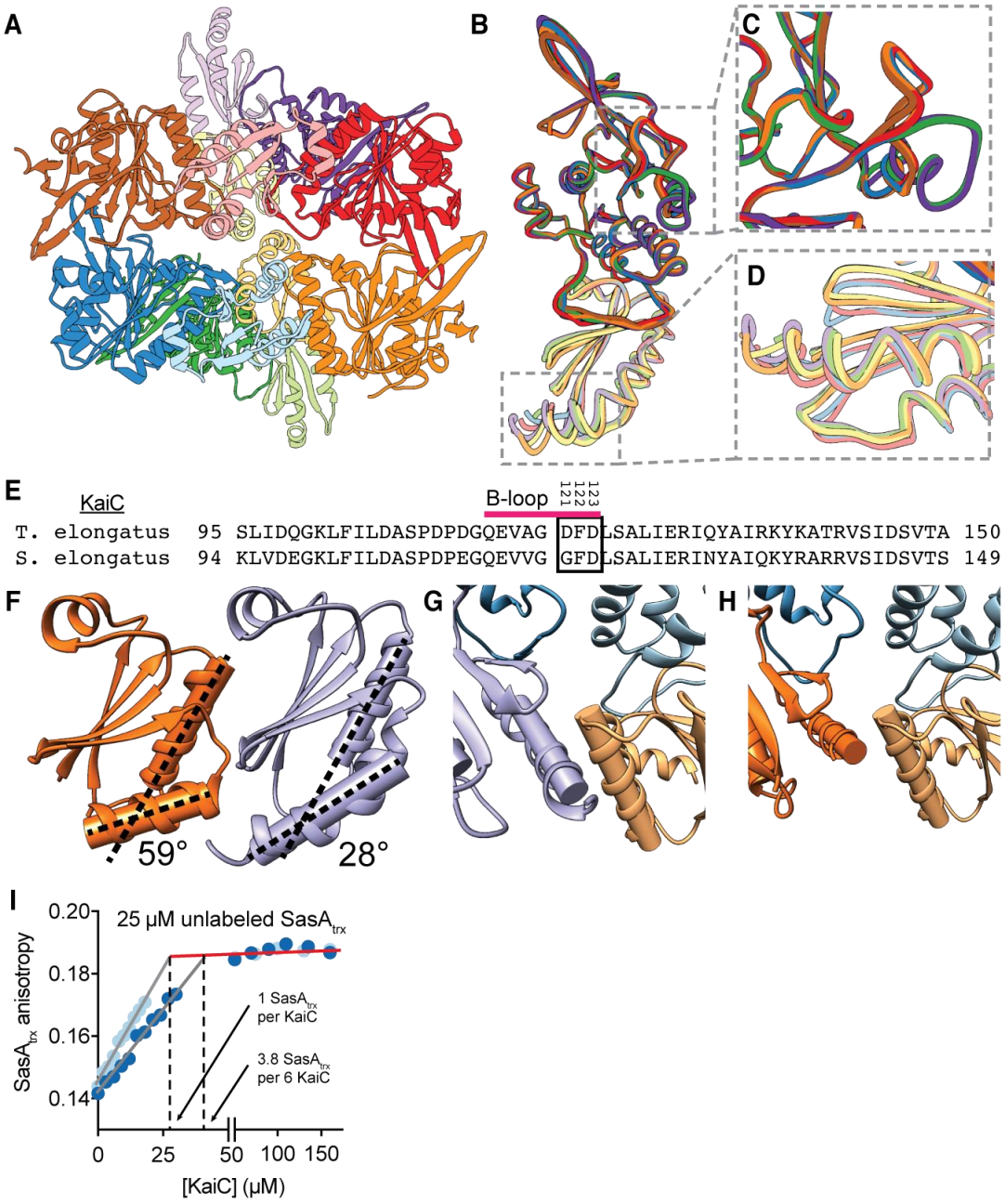

Figure 3.4. Structural details of the KaiC CI-SasAtrx complex. A) Asymmetric unit of the KaiC-C1-SasA$_{trx}$ crystal complex (PDB 6X61). Individual KaiC-CI-SasA$_{trx}$ pairs are depicted with SasA$_{trx}$ subunits in light hues and KaiC-CI subunits in dark hues. **B-D**) Modest structural heterogeneity was observed between the complexes of the asymmetric unit. Backbone overlays of the 6 KaiC CI-SasA$_{trx}$ complexes from the asymmetric unit. Small differences are highlighted by the orientation of a loop comprising residues R185-V201 on the CI domain (panel **C**) or by the orientation of the α3 helix of SasA$_{trx}$ (panel **D**), with consensus among the complexes of chains CD and KL. **E**) Multiple sequence

alignment of the B-loops (pink) and surrounding sequence from KaiC of *S. elongatus* and *T. elongatus*. Black box, residues at the shared KaiB and SasA-binding interface that were subjected to mutagenesis **F**) SasA and fsKaiB diverge structurally at the α3 helix. The α1 and α3 helices of fsKaiB (PDB: 5JWO) and $SasA_{trx}$ (PDB: 6X61) are modeled as cylindrical axes with a radius of 1.8 Å, with the inter-axis crossing angle reported. **G-H**) Orientation of the α3 helix is likely to affect binding of KaiB at the adjacent KaiC protomer. Helical orientation of the $SasA_{trx}$ (purple, panel **G**) or fsKaiB (orange, panel **H**) domain relative to the α1 helix on the adjacent KaiB molecule at the CW interface (yellow). Helices are represented as helical axes as in panel **F**. **I**) Representative saturation binding titrations of the *T. elongatus* KaiC-CI monomer or KaiC-EE hexamer in the presence of 25 µM unlabeled $SasA_{trx}$. Stoichiometry was determined assuming 1:1 binding for $SasA_{trx}$ to the KaiC-CI monomer. Stoichiometry of the saturated KaiC-$SasA_{trx}$ complex was calculated at 3.8 ± 0.3 (mean ± SD, n = 3) molecules of SasA per KaiC hexamer, consistent with measurements from native mass spectrometry.[85]

Prior studies of KaiB cooperativity demonstrated either one or six monomers of KaiB bound to the KaiC hexamer by native mass spectrometry.[83,85] Given the similarity between the SasA-KaiC and KaiB-KaiC interactions, we wondered whether SasA could influence KaiB-KaiC interactions through heterotropic cooperativity. To test this idea, we performed equilibrium binding titrations of KaiC-EE with 50 nM fluorescently-labeled KaiB probe in the absence or presence of unlabeled SasA or the fsKaiB as a secondary titrant (referred to herein as an 'additive' to the KaiC-EE titration, **Fig. 3.6A, B**). While we could not easily assess the degree of homotropic cooperativity for KaiC-EE with the KaiB probe alone under these assay conditions, low concentrations (*e.g.,* 50-100 nM) of SasA or fsKaiB significantly increased binding of the probe, demonstrating more efficient recruitment of KaiB to the KaiC hexamer. Higher concentrations of additives competed with the probe to delay its binding to the KaiC hexamer until the equivalence point where the concentration of KaiC-EE matches the additive. Interestingly, we observed a significant 'overshoot' of the typical KaiB probe anisotropy values at this point, indicative of positive heterotropic cooperativity (**Fig. 3.6A, B**). Binding of KaiB to the KaiC-CI monomer was not influenced by the addition of SasA or fsKaiB (**Fig. 3.5**), nor did SasA interact directly with KaiB in the absence of KaiC (**Fig. 3.5**), suggesting that these thioredoxin-like folds enhance the cooperative enhancement of KaiB only on the KaiC hexamer. While cooperativity operates at the KaiC hexamer level, a simplified two-site thermodynamic model was sufficient to account for the data using least-squares fitting. (**Fig. 3.6C** and 3.**5)**. We defined the heterotropic 'cooperativity index' as the fold-increase in KaiB affinity given by the ratio of equilibrium constants K_1/K_3

(=K_2/K_4). Comparison of two-dimensional (2D) titration assays with SasA or fsKaiB to simulated data representing heterotropic or homotropic cooperative binding, respectively (**Fig. 3.6D**), to a model with no cooperativity (**Fig. 3.6E**), suggests that SasA and fsKaiB similarly influence cooperative recruitment of the KaiB probe on KaiC.

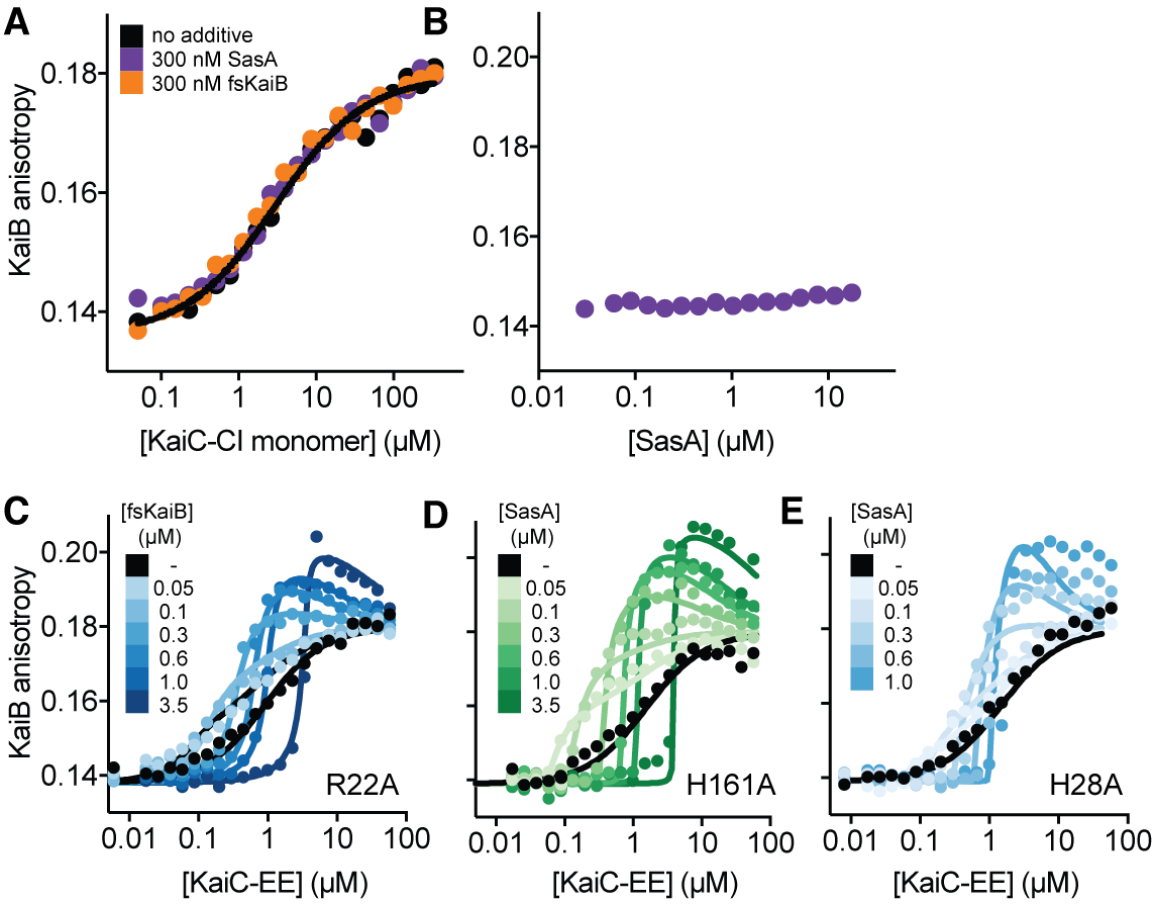

Figure 3.5. Modeling and validating heterocooperative association of KaiB with KaiC. A) SasA and fsKaiB additives do not affect binding of 50 nM fluorescently-labeled KaiB to KaiC-CI monomer. Equilibrium binding titration of fluorescently-labeled KaiB with *S. elongatus* KaiC-CI monomer (black) in the presence of 300 nM fsKaiB (orange) or full-length SasA (purple). **B**) KaiC is required for interaction between SasA and KaiB. Equilibrium titration of fluorescently-labeled KaiB with full-length SasA (purple), showing that KaiB anisotropy is unaffected without KaiC present. **C**-**E**) 2D titration assays and associated fits from the thermodynamic model for fsKaiB-R22A (panel **C**), SasA-H161A (panel **D**), and SasA-H28A (panel **E**).

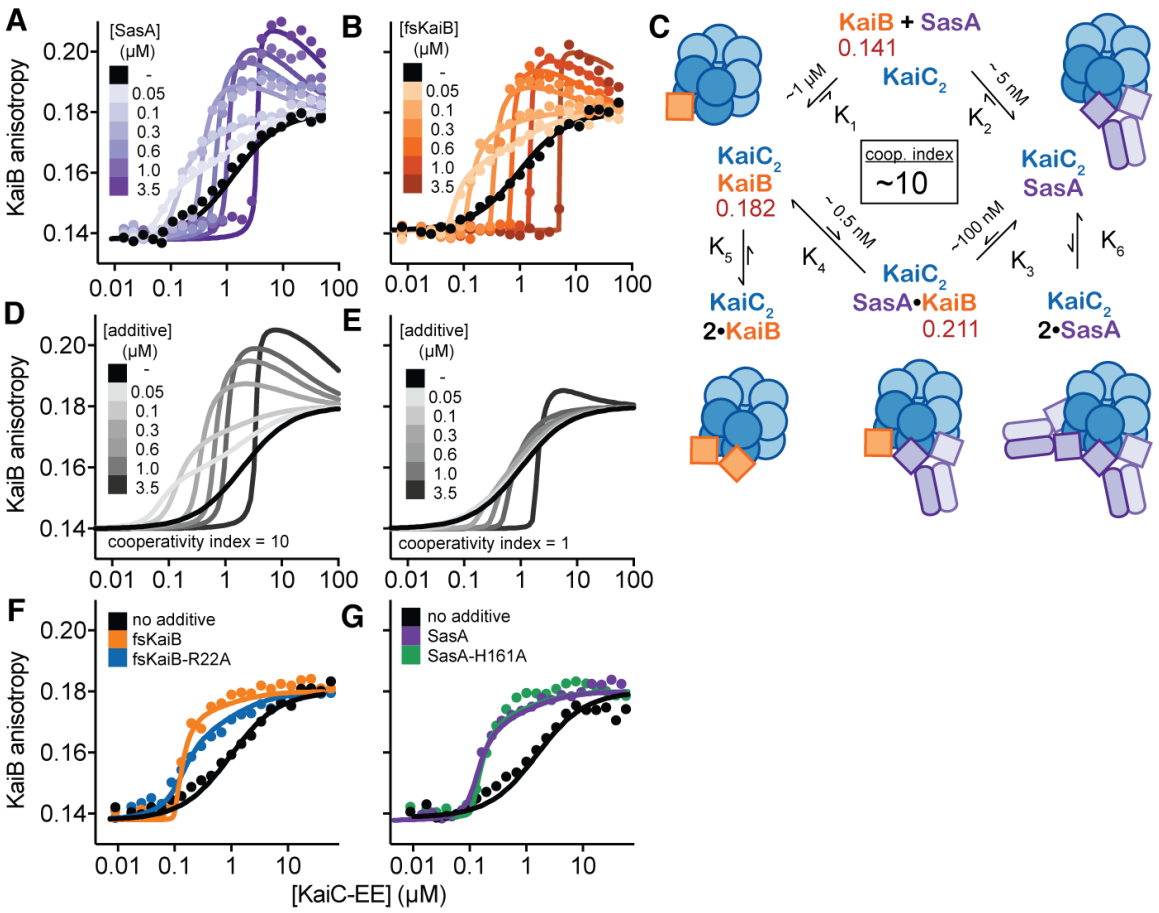

Figure 3.6. The thioredoxin-like fold of SasA and fsKaiB cooperatively recruits KaiB to the KaiC hexamer. A-B) Titrations of fluorescently-labeled KaiB with KaiC-EE in the absence (black) or presence of unlabeled full-length SasA (**A**, light to dark purple) or fsKaiB (**B**, light to dark orange). 2D titrations are shown from representative assays with global fitting to the thermodynamic model (see **Fig. 3.5**). **C**) Summary of two-site thermodynamic model for SasA heterocooperativity (darker shades) with the relevant higher-order species depicted in lighter colors. Mean equilibrium constants derived from least squares fitting of SasA 2D titration assays (n = 3); red, mean peak anisotropy values observed from global fitting of SasA 2D titration datasets. The cooperativity index is defined as the ratio $K_1/K_3 = K_2/K_4$. **D-E**) Simulated 2D titration data from thermodynamic model with a cooperativity index = 10 (**D**) or 1 (**E**). **F**) Titrations of fluorescently-labeled KaiB with KaiC-EE in the presence of 100 nM fsKaiB (orange) or fsKaiB-R22A (blue, full titration dataset in **Fig. 3.5). G**) Titrations of fluorescently-labeled KaiB with KaiC-EE in the presence of 100 nM SasA (purple) or SasA-H161A (green, full titration dataset in **Fig. 3.5**).

To explore the role of intersubunit interactions in KaiB cooperativity, we first turned to the KaiB-R22A mutant originally identified in *Anabaena*,[133] which reduces the apparent affinity of KaiB for KaiC. This residue has been implicated in KaiB cooperativity[85] and is situated at the KaiB-KaiB interface in the KaiBC hexamer structure.[56] When this mutant was tested in our cooperativity assay, we observed a decrease in the cooperative recruitment of the KaiB probe relative to fsKaiB (**Fig. 3.6G** and **3.5**), demonstrating that inter-subunit KaiB-KaiB interactions play a role in this cooperative recruitment process. Moreover, although SasA kinase activity is required for its role in regulating circadian rhythms of transcriptional output *in vivo*,[132] we found that the kinase-dead SasA mutant H161A is as effective as wild-type SasA in stimulating cooperative recruitment of KaiB to KaiC *in vitro* (**Fig. 3.6G**).

To see whether SasA's role as a positive regulator of KaiBC association is important for its function *in vivo*, we set out to identify point mutations at potential cooperativity interfaces. We used crystal structures of $SasA_{trx}$-CI and fsKaiB-C1 to model a $SasA_{trx}$-KaiC-KaiB complex to examine potential $SasA_{trx}$-KaiB interactions along the adjacent clockwise (CW) and counterclockwise (CCW) interfaces of the hexamer (**Fig. 3.7A**). Within this framework, we investigated a number of SasA mutations near the CW and CCW interface to see how they would influence cooperative recruitment in KaiB binding assays (**Fig. 3.8**). Using analysis of 2D titration data with the mutants, we found a decrease in the cooperativity indices for the individual mutants H28A (CCW interface) or Q94A (CW interface) that was further decreased in the H28A/Q94A double mutant (**Fig. 3.7B**). The H28A/Q94A displayed a striking lack of heterocooperativity in the 2D titration assay (**Fig. 3.7C** compared to simulated data in **3.6E**), demonstrating that SasA-KaiB interactions at both interfaces are important for cooperative recruitment.

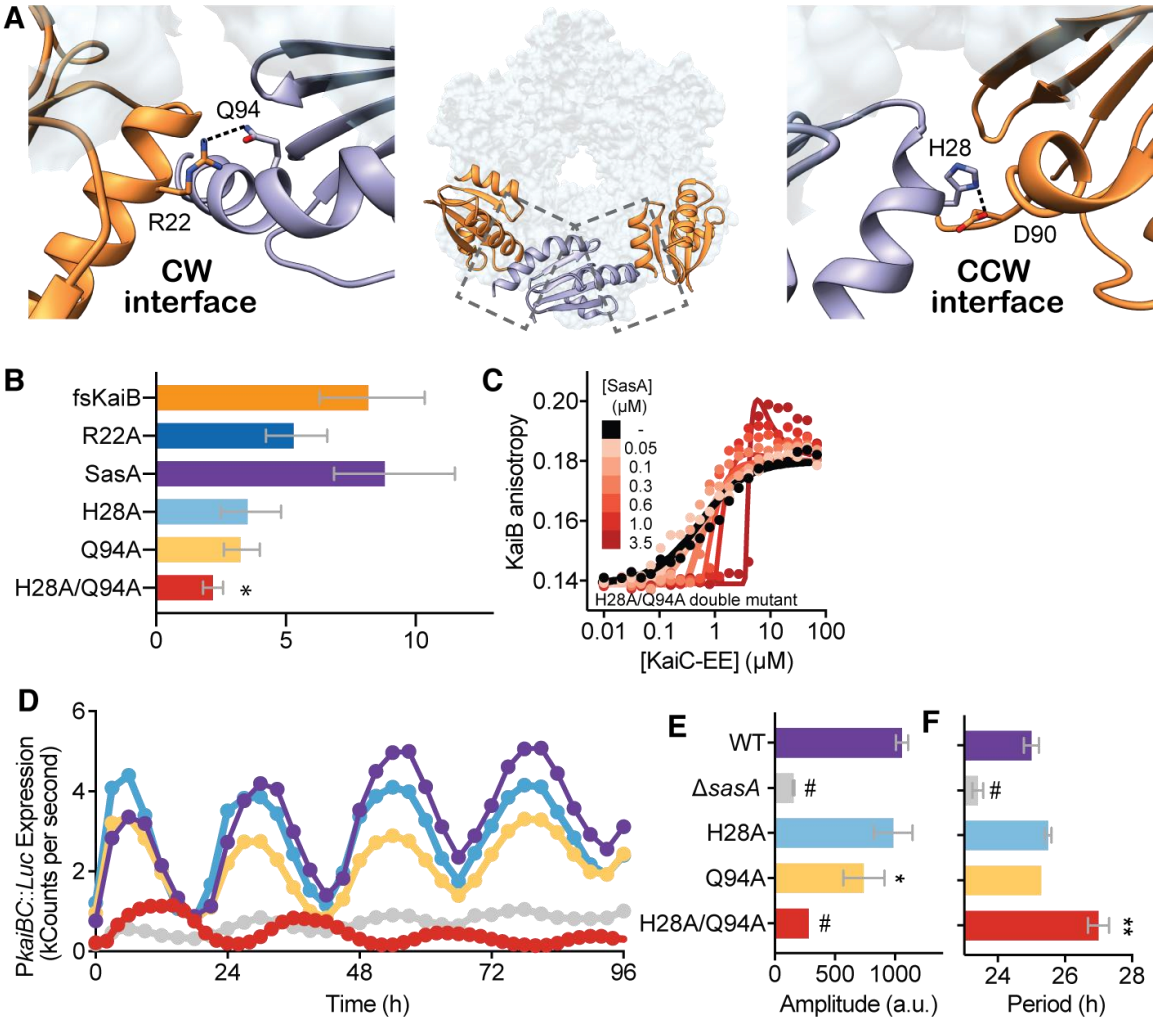

Figure 3.7. SasA-KaiB interactions mediate cooperative recruitment of KaiB *in vitro* and sustain robust circadian rhythms *in vivo*. A) Structural model of KaiB:SasA interactions on KaiC from KaiC-CI subcomplexes with fsKaiB (orange, PDB:5JWO) or $SasA_{trx}$ (purple, PDB:6X61) modeled onto adjacent subunits of KaiC (light blue) of the KaiBC hexamer (PDB:5JWQ). Key residues at clockwise (CW) and counterclockwise (CCW) interfaces are modeled as *S. elongatus* variants based on the alignment in **Fig. 3.8**. Dotted lines, polar contacts predicted by hybrid structural model. **B**) Cooperativity indices for KaiB and SasA mutants from thermodynamic model. Bars represent median and 95% confidence intervals from Monte Carlo analysis from n = 2-3 full 2D datasets. See Supplemental Methods for more details. Statistical analysis was performed on variance between median Monte Carlo values between replicates (*, $P < 0.05$) **C**) Representative 2D titration of fluorescently-labeled KaiB with KaiC-EE in the absence (black) or presence of SasA-H28A/Q94A (light to dark red). **D**) Representative timecourse of bioluminescence driven by P*kaiBC* from *S. elongatus* cultures entrained under 12-h LD cycles for 48-hr and subsequently

allowed to free run in LL. Raw luminescence curves were fit to a sine function to extract amplitude (**E**) and free running circadian period (**F**) for each strain. Error bars depict standard deviation among replicate cultures (n = 6-12); when error bars are not visible, they were smaller than could be depicted. Symbols indicate significance (**, $P < 0.01$; #, $P < 0.0001$) from one-way ANOVA of mutants relative to wild-type *S. elongatus*.

Figure 3.8. Multiple sequence alignment of KaiB and SasAtrx from S. and T. elongatus. A) Multiple sequence alignment of N-terminal (non-fold-switch) and C-terminal (fold-switch) halves of KaiB (orange box) and the structurally analogous SasAtrx (purple box) from S. elongatus and T. elongatus. Red arrows (β-sheets) and blue lines (α-helices) indicate secondary structure from the KaiB tetramer (PDB: 2QKE), fsKaiB monomer (PDB: 5JWO) and SasAtrx from the KaiC-CI-SasAtrx structure (PDB: 6X61) reported here. Mutations tested in this study are depicted in bold.

To see if the SasA-induced heterocooperativity we observed *in vitro* influences circadian rhythms *in vivo*, we introduced these substitutions into SasA using CRISPR/Cas12a and monitored bioluminescence from a P*kaiBC* luciferase reporter in constant light (LL) after synchronization in 12-h light:12-h dark cycles (**Fig. 3.7D** and **3.9**). Modest effects on amplitude were seen with the individual H28 or Q94 alanine substitutions on their own, but when these were combined in the H28A/Q94A mutant, the amplitude of circadian rhythms was decreased to an extent that was similar to the SasA knockout strain (Δ*sasA*, **Fig. 3.7E**). Furthermore, we observed a 2-h increase in period in the double-mutant strain

(**Fig. 3.7F**), suggesting that heterocooperative interactions at the CW and CCW interfaces are redundant, but crucial for circadian timekeeping *in vivo*.

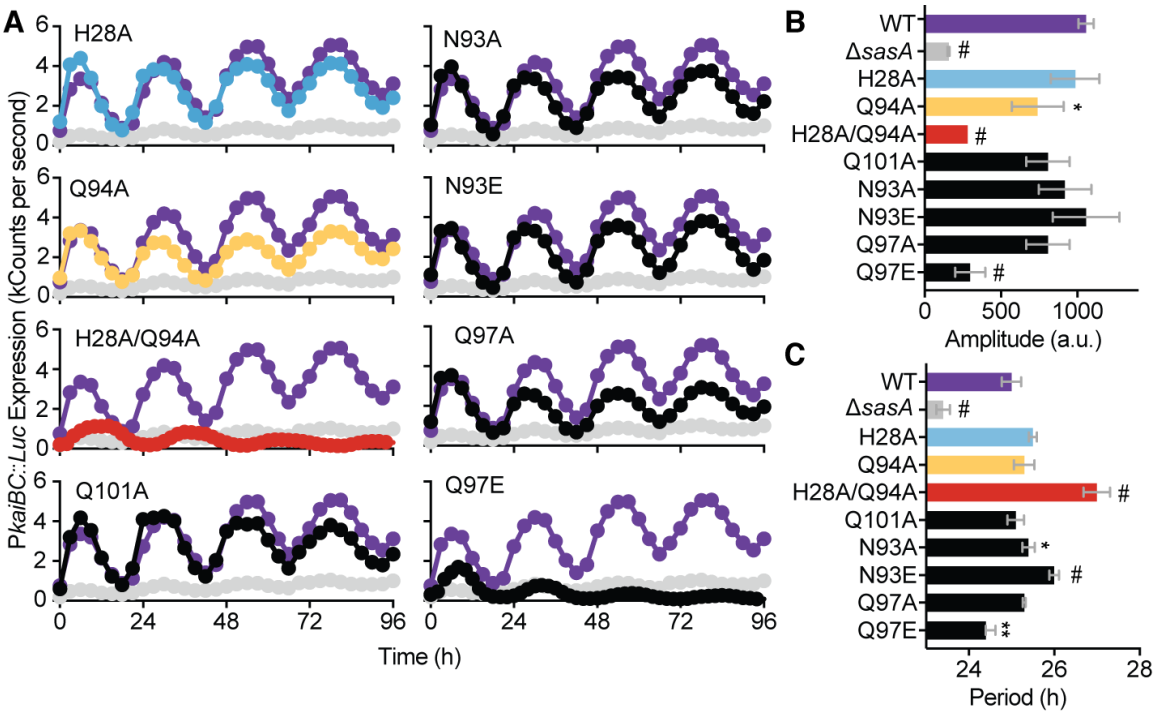

Figure 3.9. Mutations at the SasA-KaiB cooperativity interface alter circadian rhythms in S. elongatus. A) Bioluminescence traces produced by *S. elongatus* strains based on expression of firefly luciferase driven by the clock-controlled *kaiBC* promoter. Wild type (WT) and Δ*sasA* strains are compared to markerless CRISPR/Cas12a-edited *sasA* mutants, with the relevant amino acid substitutions indicated. Graphs display the average of 6-12 wells, with standard deviation omitted for better visibility of multiple traces. WT (purple) and mutants, colored as in panel **B**. **B-C**) Analysis of raw bioluminescence data was performed using BioDare2 to calculate the signal amplitude (in arbitrary units, a.u.) (panel **B**) and period length (panel **C**), with the average and standard deviation reported for 6-12 replicates. One-way ANOVA was performed to identify significant changes (*, $P < 0.05$; **, $P < 0.01$; #, $P < 0.0001$) in amplitude and period length relative to wild type.

SasA was originally described as an amplifier of circadian rhythms, critical for maintaining robust rhythmicity by controlling rhythmic transcription of the *kaiBC* cluster.[132] To test whether this amplitude-enhancing effect might be due in part to SasA regulation of KaiB binding to KaiC, we turned to the FP-PTO assay to

explore the effects of SasA on robustness of the core circadian oscillator in the absence of transcription-translation feedback that occurs *in vivo*.[23] First, we measured the dependency of this oscillator assay on the concentrations of KaiA and KaiB, comparing data from the FP-PTO to a prior study using rhythms of KaiC phosphorylation to find a striking convergence in our results (**Fig. 3.10A, B**).[124] Because the FP-PTO is monitored non-invasively in 384-well format, we could follow the oscillator under different conditions for many days longer than is practical for the KaiC phosphorylation assay that is analyzed by SDS-PAGE. Generally, oscillator amplitude and period were highly sensitive to KaiA concentrations, and while there was a clear requirement for a minimal concentration of KaiB, the period was quite robust to excess KaiB relative to KaiC (**Fig. 3.10C-E**). Although the oscillator functioned at low amplitude for several days with a normal circadian period with one-half stoichiometry of KaiB relative to KaiC (i.e., 1.75 μM KaiB for 3.5 μM KaiC),[124] these rhythms rapidly damped (**Fig. 3.10B**). KaiB is present in only modest excess over KaiC *in vivo*,[75,121] which suggests that the ability of KaiC to cooperatively recruit and bind six KaiB monomers is critical to sequester KaiA stably during negative feedback in the PTO and is thus a determinant of clock robustness. Consistent with this idea, the amplitude of the PTO decreased as the concentration of KaiA approached or exceeded that of KaiC (**Fig. 3.10A, C** and [93,124]).

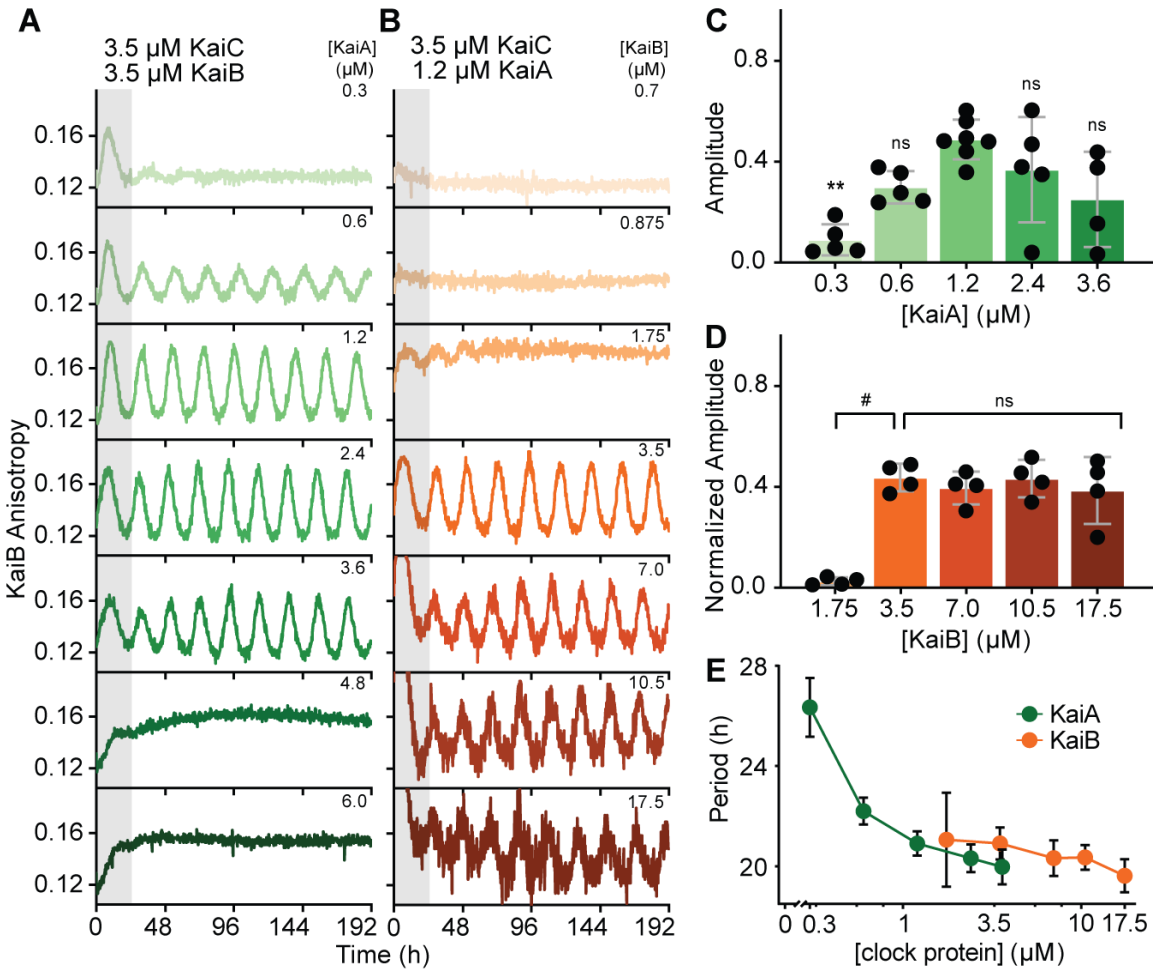

Figure 3.10. Robustness and period of the oscillator depend in different ways on the concentrations of KaiA and KaiB. A) The FP-PTO *in vitro* with standard conditions of 3.5 μM KaiC, 3.5 μM KaiB (including 50 nM fluorescently-labeled KaiB as a probe) and titrations of KaiA from 0.3-6.0 μM. Representative assay from n =3 shown; the first 24-hr period after release into constant conditions is marked in gray. **B**) The FP-PTO with standard conditions of 3.5 μM KaiC, 1.2 μM KaiA and titrations of KaiB from 0.7-17.5 μM (including 50 nM fluorescently-labeled KaiB as a probe). **C-D**) Amplitude analysis of KaiA titrations (**C**, data from panel **A**) or KaiB titrations (**D**, data from panel **B**). Amplitudes for KaiB titrations were normalized to account for increasing ratio of unlabeled KaiB to fluorescently-labeled KaiB. Reaction conditions that could not be fit by FFT-NLLS analysis, and therefore did not oscillate, were not included. Data are shown as mean ± SD (n ≥ 3). One-way ANOVA with Dunnett's multiple comparisons test was used for comparison between groups: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. **E**) Period of the FP-PTO plotted against KaiA (green) or KaiB (orange) concentrations. Data are shown as mean ± SD (n ≥ 3).

Because of its ability to enhance formation of the KaiBC complex, SasA has the potential to improve robustness of the PTO under limiting concentrations of KaiB by recruiting a stable, stoichiometric complement of KaiB to KaiC hexamers to sequester KaiA. To explore how SasA influences KaiB binding in the context of an oscillator, we set up FP-PTO assays under typical *in vitro* oscillator conditions[31] as well as under limiting concentrations of KaiB relative to KaiC. Low anisotropy levels for labeled KaiB under the limiting condition of only 0.875 μM KaiB (**Fig. 3.11A**, left column) demonstrated incomplete KaiB binding to the KaiC hexamer. The addition of full-length SasA up to 1 μM increased KaiB anisotropy levels, demonstrating that SasA enhanced KaiB binding to KaiC in the presence of KaiA, even without a functional oscillator. However, as the concentration of SasA approached or exceeded that of KaiC, anisotropy values for KaiB dropped, indicating that SasA outcompeted KaiB for binding to the KaiC hexamer as observed before.[81,134,135] When KaiB was present at one-half stoichiometry with respect to KaiC (1.75 μM KaiB for 3.5 μM KaiC), the addition of SasA restored robust oscillations to the FP-PTO (**Fig. 3.11A**, middle column). This enhancement appears to be almost catalytic, as only 100 nM SasA was sufficient to rescue damping (**Fig. 3.11B**) and restore amplitude (**Fig. 3.11C**) to wild-type levels. Increasing SasA concentrations to 1 μM under these conditions did not influence amplitude any further, while higher concentrations of SasA outcompeted KaiB binding to attenuate oscillations in the FP-PTO (**Fig. 3.11C**).

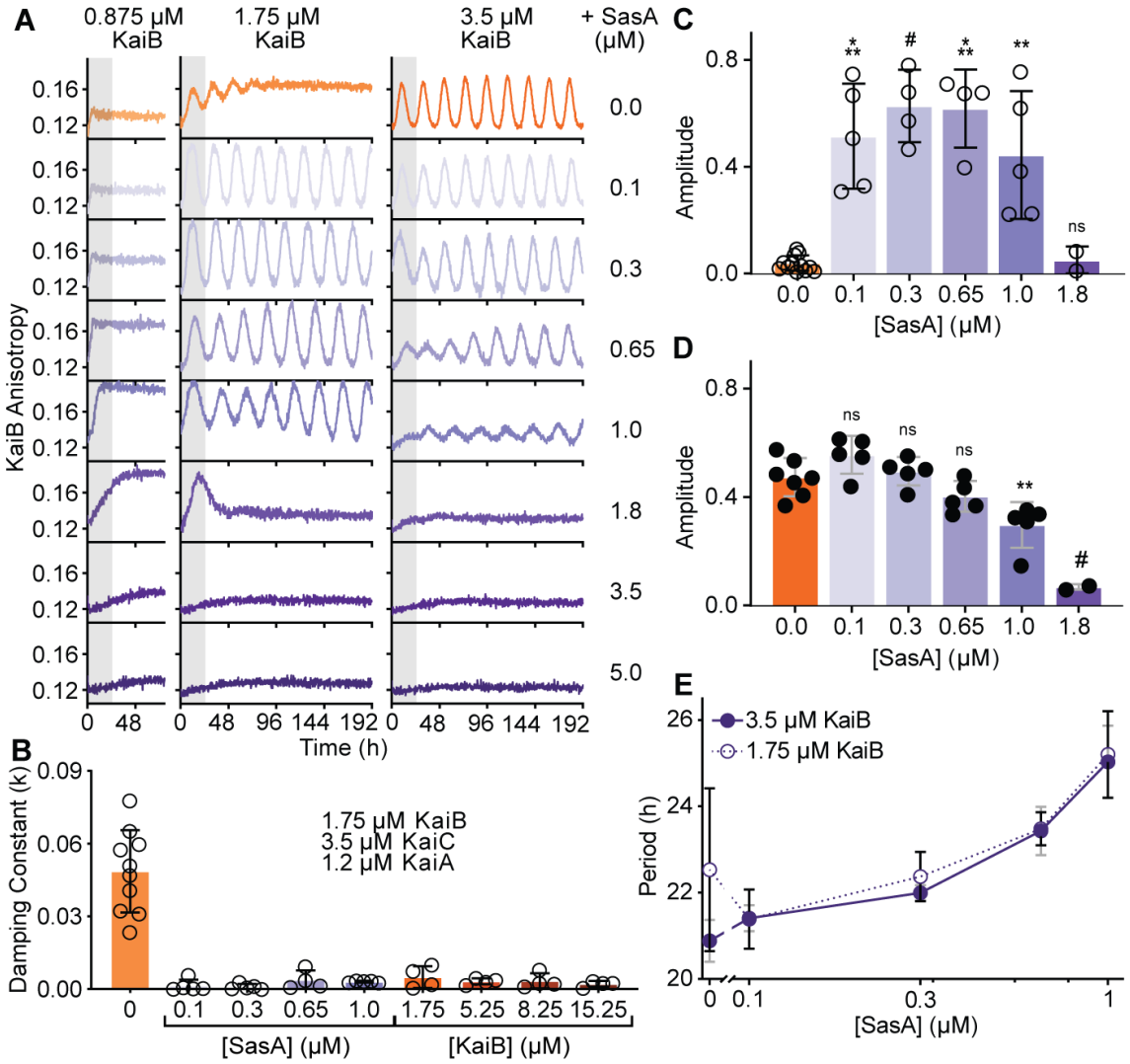

Figure 3.11. SasA dynamically influences robustness of the post-translational oscillator *in vitro*. A) The FP-PTO with 3.5 μM KaiC, 1.2 μM KaiA, and the indicated concentrations of KaiB (including 50 nM fluorescently-labeled KaiB as a probe) with titrations of full-length SasA 0.1-5.0 μM. Representative assay from n ≥ 3 shown; the first 24-hr period after release into constant conditions is marked in gray. **B**) Damping constants of the FP-PTO with 1.75 μM KaiB (including 50 nM fluorescently-labeled KaiB as a probe) in the presence of added SasA (purple) or KaiB (orange). **C-D**) Amplitude of the FP-PTO prepared with 1.75 μM KaiB (**C**, open circles) or 3.5 μM KaiB (**D**, closed circles) in the presence of SasA (light to dark purple). **E**) Period of the FP-PTO prepared with 1.75 μM KaiB (open circles) or 3.5 μM KaiB (closed circles) with SasA (light to dark purple). Reaction conditions that could not be fit by FFT-NLLS analysis, and therefore did not oscillate, were not included. Damping constant, period, and amplitude data are representative of four or more independent experiments, are

presented individually and as the mean ± SD. One-way ANOVA with Dunnett's multiple comparison test was used for comparison between groups: ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$.

Under standard oscillator conditions with stoichiometric KaiB and KaiC, the presence of SasA up to 0.65 μM did not markedly influence the oscillator (**Fig. 3.11A**, right column), whereas at increased concentrations of SasA, oscillator amplitude diminished until KaiB was completely outcompeted for KaiC binding (**Fig. 3.11B, D**). This suggests that the competitive effects of SasA may be enhanced as the concentration of KaiB equals KaiC. Moreover, the sharp drop in amplitude of the FP-PTO suggests that the functional switch from cooperativity to competition occurs around ~1 μM SasA depending on the exact oscillator conditions; interestingly, this ultrasensitive setpoint is close to the concentration of SasA *in vivo* estimated by quantitative western blotting (0.58 ± 0.07 μM from [58]). In all cases where conditions were sufficient to generate a stable PTO, the period was lengthened similarly by addition of SasA (**Fig. 3.11E**). Therefore, in addition to regulating rhythms of transcriptional output from the Kai-based PTO,[132] SasA also works to directly modulate KaiB association with KaiC to control formation of the nighttime repressive state and robustness of the PTO itself.

The other circadian output kinase, CikA, also contributes directly to robustness of the PTO by enhancing rhythms under limiting concentrations of KaiA.[103] Using the FP-PTO assay, we observed that addition of CikA moderately enhanced low amplitude rhythms under limiting concentrations of KaiA while shortening the period (**Fig. 3.12**). The pseudo-receiver (PsR) domain of CikA binds to the same site on KaiB in the nighttime complex as KaiA does,[56] and their competition *in vivo* is made evident by the phenotypes of a *kaiA* deletion mutant [123]. By competing KaiA out of the repressive complex, CikA promotes the activating potential of KaiA to stimulate KaiC phosphorylation.[72,103] Either the isolated PsR domain or full-length CikA caused similar concentration-dependent decreases in period length in the FP-PTO assay, while the isolated thioredoxin-like domain of SasA was much less effective at lengthening the period compared to full-length SasA, likely due to avidity effects (**Fig. 3.3** and 3.**13**).

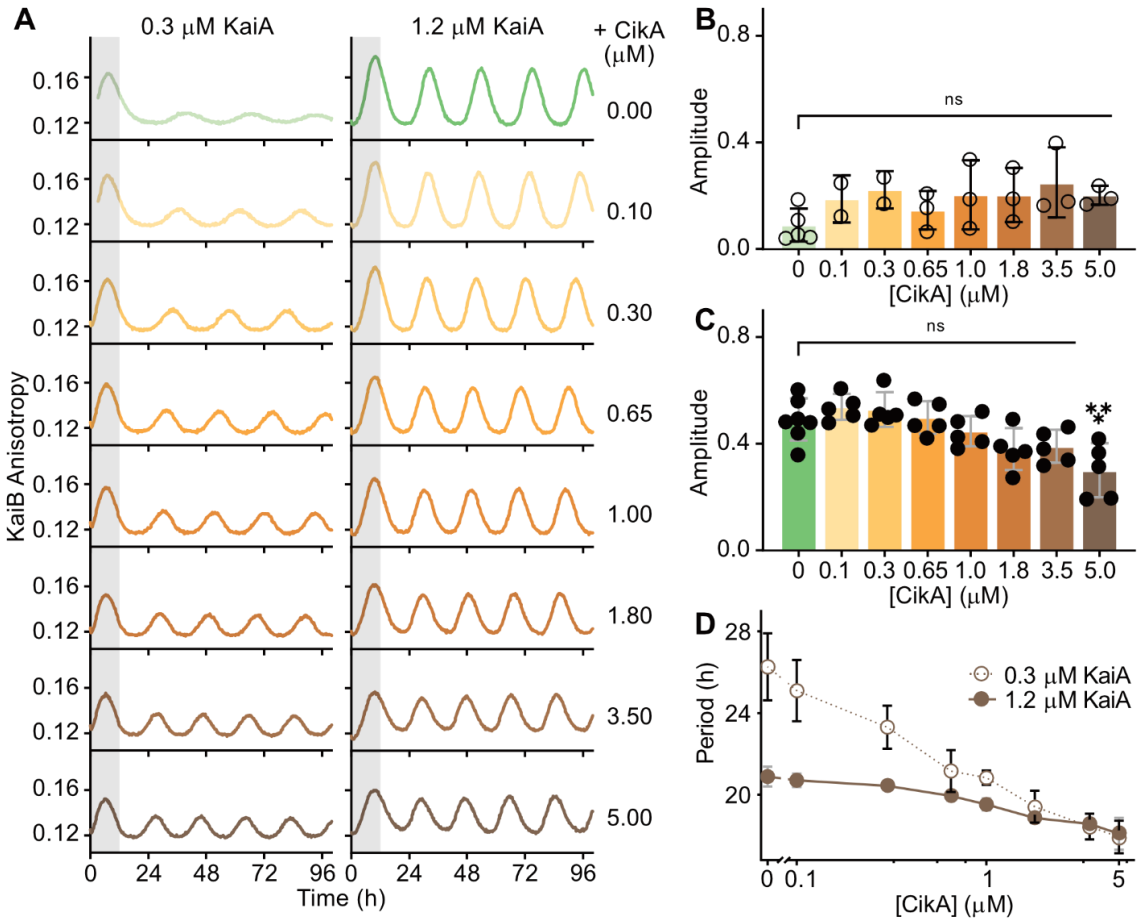

Figure 3.12. Enhancement of oscillator robustness by CikA under limiting concentrations of KaiA. A) The FP-PTO under conditions of 3.5 μM KaiC, 3.5 μM KaiB (including 50 nM fluorescently-labeled KaiB as a probe), and either 0.3 μM or 1.2 μM KaiA as indicated with titrations of CikA from 0.1 – 5.0 μM. Representative assay from n ≥ 2 shown; the first 12-h period after release into constant conditions is marked in gray. **B** and **C**) Amplitude for FP-PTO assays prepared under different KaiA concentrations of (panel **B**) 0.3 μM (open circles) or (panel **C**) 1.2 μM (closed circles) KaiA in the absence (green) or presence of CikA (light yellow to brown). ANOVA was used to compare amplitudes of the FP-PTO under the two KaiA concentrations in the absence of CikA versus the indicated concentrations of CikA: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. **D**) Period values for FP-PTO assays prepared with 0.3 μM (open circles) or 1.2 μM (closed circles) KaiA in the absence and presence of CikA (brown). Period and amplitude data are representative of two or more independent experiments, presented as the mean ± SD.

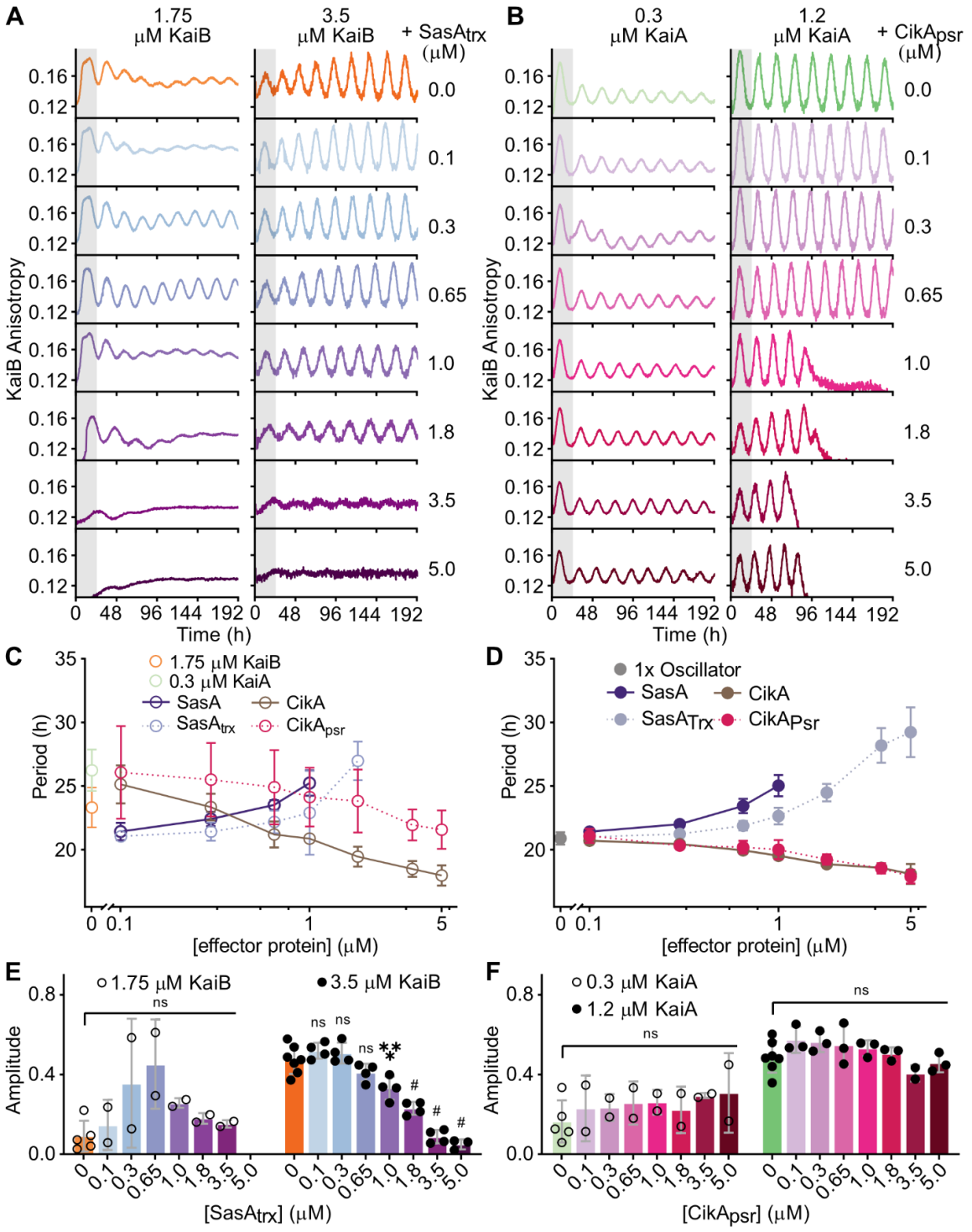

Figure 3.13. Domain truncation studies on SasA and CikA oscillator effects. A-B) The FP-PTO under conditions of 3.5 μM KaiC, 50 nM fluorescently-labeled KaiB as a probe, and either (panel **A**) standard 1.2 μM KaiA with 1.75 μM or 3.5 μM KaiB, or (panel **B**) standard 3.5 μM KaiB with 0.3 μM or 1.2 μM KaiA as indicated with titrations of truncated domain (panel **A**) $SasA_{trx}$ or (panel **B**) $CikA_{psr}$

from 0.1 – 5.0 μM. Representative assay from n ≥ 2 shown; the first 24-h period after release into constant conditions is marked in gray. **C-D**) Period values for FP-PTO assays prepared with deficient (panel **C**) 1.75 μM KaiB and (panel **D**) 0.3 μM KaiA (open circles) or standard oscillator concentrations (panel **C**) 3.5 μM KaiB and (panel **D**) 1.2 μM KaiA (closed circles) in the absence and presence of full-length (solid connecting lines) and truncated additive proteins (dotted connecting lines). Period and amplitude data are representative of one or more independent experiments with duplicate samples, presented as the mean ± SD. **E-F)** Amplitude for FP-PTO assays prepared under different core KaiB and KaiA concentrations of (panel **E**) deficient (open circles) or (panel **F**) standard (closed circles) in the absence or presence of truncated additive proteins. Data are shown as mean ± SD (n ≥ 2 with duplicate samples). Analysis of variance (ANOVA) was used to compare amplitudes of the FP-PTO under the two KaiB and KaiA concentrations in the absence of truncated domains $SasA_{trx}$ and $CikA_{psr}$, respectively, versus the indicated concentrations of those additive proteins: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$.

The FP-PTO assay implemented here has uncovered roles for the output kinases as accessory components of the oscillator, expanding its functionality beyond the narrow concentrations and ratios that are tolerated in the traditional *in vitro* oscillator.[124] This extended oscillator reveals how the clock maintains consistency *in vivo* throughout rhythmic changes in oscillator components that occur as part of transcription-translation feedback[75,136] and protein turnover,[137,138] as well as providing an experimental platform for integrating the oscillator with the upstream and downstream components with which it interacts.

Materials and Methods

Cloning of constructs

PCR-mediated mutagenesis was performed on the pET-28b vector utilizing Nde I/Hind III cut sites as described previously.[76] Full-length *sasA* was cloned into the pHis-Gβ1 parallel expression vector using EcoRI/NotI cut sites.[139,140] All constructs are listed in **Table 3.2**. Point mutations were introduced using long-range PCR with overlapping primers.[141]

Protein expression and purification

Protein expression for most constructs was carried out in BL21(DE3) *E. coli* (Novagen) and purified by Ni-NTA affinity chromatography and size-exclusion chromatography as described previously.[76] All buffers for KaiC purification included 1 mM ATP and 5 mM $MgCl_2$ added as described Chang et al..[76] Fluorescence labeling of KaiB, $SasA_{trx}$, and $CikA_{psr}$ constructs was done with 6-iodoacetamidofluorescein (6-IAF, Invitrogen) as described previously.[81]

After purification of His-Gβ1-tagged full-length SasA constructs over Ni-NTA resin using the standard protocol described in Chang et al.,[76] the protein was cleaved overnight at 4°C with TEV protease a final concentration of 0.1 mg/mL. SasA was subsequently resolved from the His-Gβ1 tag on a Sephadex 200 size-exclusion column (GE Healthcare) equilibrated with 20 mM Tris pH 7.4, 150 mM NaCl.

Fluorescence Polarization - Post Translational Oscillator (FP-PTO) assays

Fluorescence anisotropy oscillator data were collected *in vitro* on either a CLARIOstar Plus (BMG) or Spark 10M (TECAN) microplate reader. The fluorescein channel was used for all data collection ($\lambda_{excitation}$, 482 ± 8 nm; $\lambda_{emission}$, 530 ± 20 nm). Fluorescence anisotropy was monitored from each well in the 384-well plate and recorded every 15 minutes, with time zero representing 3-5 minutes following the addition of KaiC to oscillation reactions. See **Table 3.3** for specific experimental conditions for each run.

FP-PTO data quantification and statistical analyses

Fluorescence anisotropy readings from FP-PTO assays were collected in MARS Data Analysis Software or SparkControl Software for experiments run on the BMG CLARIOstar Plus or TECAN Spark 10M, respectively. All data were analyzed in the online BioDare suite by FFT-NLLS[128,129] (https://biodare2.ed.ac.uk/welcome). Prior to analysis, fluorescence anisotropy rhythms were baseline detrended and normalized to [-1, 1] with mean of zero. The first 12-h of data were disregarded for quantification of period, amplitude, and phase. Period (**Table 3.4**) and amplitude (**Table 3.5**) analysis in **Fig. 3.10, 3.11, 3.12,** and **3.13** were assessed by ordinary one-way ANOVA with Dunnett's multiple comparison tests in Prism 8 (Graph Pad). The significance values and the number of independent experiments for each experimental group are reported in the corresponding figure legends.

Comparisons of the effects of added SasA or CikA on period (**Table 3.6**) and amplitude (**Table 3.7**) under different concentrations of the core clock proteins KaiA and KaiB presented in **Table 3.3** were determined by ordinary two-way ANOVA with Dunnett's multiple comparison tests in Prism 8 (GraphPad). Normalized fluorescence anisotropy rhythms were plotted in conjunction with nonlinear regression least squares cosinor fit in Prism 8 in **Fig. 3.1**, while raw fluorescence anisotropy data were plotted with Origin Student 2019b (Origin Lab) in **Fig. 3.10, 3.11, 3.12,** and **3.13**. Raw anisotropy data were baseline corrected using an unbound labeled-KaiB reaction well as a reference in each independent experiment. The phase diagram in **Fig. 3.1C** was prepared using phase values resulting from analysis by BioDare2[128,129] as described above.

The damping constant, k, was determined in Prism 8 (GraphPad) using a nonlinear least squares regression cosinor fit:

$$y = m * x + amplitude * e^{-kx} * \cos\left[\left(2\pi * \frac{x}{period}\right) + phase\right]$$

where y is the signal, x the corresponding time, amplitude is the height of the peak of the waveform above the trend line, k is the decay constant (such that 1/k is the half-life), period is the time taken for a complete cycle to occur and phase is the shift in x relative to a cosinor wave.

KaiC phosphorylation assay

KaiC phosphorylation assays were performed *in vitro* as previously described.[84] Densitometry was used to quantify KaiC phosphorylation using ImageJ FIJI (NIH). Quantification of phases for phosphorylation densitometry rhythms was assessed in the online BioDare2 suite[128,129] as described above.

Crystallization of monomeric *T. elongatus* C1 domain in complex with *T. elongatus* $SasA_{trx}$

A monomeric mutant of *T. elongatus* KaiC-CI domain (see **Table 3.2** for details) was incubated at 250 μM with an excess of *T. elongatus* $SasA_{trx}$ (460 μM) overnight in 20 mM Tris pH 7.0, 150 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$ and 1 mM ATP at room temperature. The complex was subsequently purified by size-exclusion chromatography on a Sephadex 70 column (GE Healthcare) equilibrated in the same buffer, but with $MgCl_2$ and ATP concentrations reduced

to 0.5 mM. The complex was mixed in a 1:1 ratio to a final concentration of 10.8 mg/mL with the crystallization buffer containing 1.26 M NaH_2PO4, 0.54 M K_2HPO_4 (pH unadjusted, total PO_4 concentration 1.8 M), 0.1 M Glycine (added from a 1 M solution adjusted to pH 10.5) and 0.2 M $Li_2(SO_4)_2$. Crystals formed over 10 days at 22 °C using the hanging drop method. The flat, plate-like crystals were then frozen in liquid nitrogen after soaking in cryoprotectant composed of the crystallization buffer plus 20% (v/v) glycerol.

Structure determination and refinement

Single crystal diffraction data were collected with a wavelength of 1 Å on the 23-ID-D X-ray source at the Advanced Photon Source at the Argonne National Laboratory. Data were processed and scaled using MOSFLM[142] and Aimless.[143] Phases were solved by molecular replacement with the structure of *T. elongatus* KaiC-CI monomer in complex with fsKaiB (PDB 5JWO) using Phaser.[144] Refinement and model building were performed using Phenix[145] and Coot.[146] See **Table 3.1** for crystal and refinement statistics. Structural figures were made using UCSF Chimera[147,148] and ChimeraX.[149]

Equilibrium binding assays

Binding titrations were performed in 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM ATP, 1 mM $MgCl_2$ and 0.1 % (v/v) Tween-20. Fluorescein-labeled KaiB or $SasA_{trx}$ probes were present at 50 nM, while the titrant was diluted serially in 1/3-fold increments. Serial dilutions were performed in a 384-well plate before sealing with tape and incubating at room temperature overnight (9-15 h). Fluorescence polarization anisotropy measurements were subsequently collected on a Synergy2 plate reader (BioTek). Replicate measurements were collected and averaged for each well (20). For 2D titration assays looking at the effect of an additive on KaiB binding to KaiC, fsKaiB or SasA additives were included in both KaiC and diluent buffer to maintain a constant concentration. Diluent was added to the 384-well plate using a single channel pipettor, and additives were mixed into the KaiC stock last and diluted within 10 minutes. See thermodynamic modeling of binding equilibria below for more information.

Thermodynamic modeling of binding equilibria

The fluorescence anisotropy titrations outlined above involve cooperative and competitive reactions and span a wide range of concentrations, such that free ligand concentrations cannot be approximated by the total added concentration.

Consequently, these data cannot be analyzed by fitting to standard analytical equations.[150] Fitting the data to the profiles simulated by a model avoids this problem but introduces others in terms of the complexity of the model that is required for the fit. Initially, we attempted to fit to a general hexameric model for KaiC but found there were too many parameters to reach convergence. When simplified to a dimer model, the fits were reasonably robust and showed no systematic deviations. Nevertheless, such a simplified model required positive heterotropic or homotropic cooperativity between KaiB and additives such as SasA and fsKaiB, respectively, as well as competition between these additives. Thus, a dimer model captures the essence of the interaction, although how this relates in detail to cooperativity within the KaiC hexamer remains in question.

Least-squares fitting analysis to models was performed using DynaFit (BioKin).[151] Statistical analysis was performed using the Monte Carlo routine. Cooperativity indices (described by $K_1/K_3 = K_2/K_4$) were calculated for each simulation (n = 1000) and median and 95% confidence intervals taken as ranks 500, 25 and 975 (respectively) in the n = 1000 simulation. Where replicate measurements are reported, the values of median or 95% confidence boundaries were averaged amongst the replicates.

In order to reduce the number of parameters of the fit, the binding of KaiB alone was initially modeled without any homotropic cooperativity by assigning $K_5 = 4*K_1$, as is appropriate for the macroscopic equilibrium constants for two-site independent binding. When K_5 was floated, a slightly improved fit was obtained with a returned $K_5 < K_1$, indicative of homotropic cooperativity, but K_5 was not robustly defined. The value of the heterotropic cooperativity index in the presence of additives increased when K_5 was floated, however, we report fits where K_5 was defined as $4*K_1$ for simplicity, which gives a minimal estimate of the heterotropic cooperativity index.

Triplicate 2D titrations were collected with SasA to optimize the analysis, and showed some variability that was ameliorated by floating additive concentrations at the 3 highest additive concentrations. The averages from these fits were used for SasA and KaiB variant 2D titration datasets when analyzing 300 nM data, where additive concentrations were also allowed to float. Little variation was seen in the experimental anisotropy values determined for fluorescently-labeled KaiB alone or the final peak values for the KaiB-KaiC complex, though the average peak experimental anisotropy values of putative ternary complexes seeded by

heterocooperativity differed modestly between the SasA and fsKaiB variants (KaiB peak anisotropy = 0.211 for SasA or 0.205 for fsKaiB).

Size-exclusion chromatography-multiangle light-scattering (SEC-MALS) assays

SEC-MALS assays were performed at room temperature using a silica-based size-exclusion column (particle size 5 µm, pore size 500 Angstrom, 4.6 mm ID, Cat. No. WTC-050N5, Wyatt Technologies) to resolve the oligomeric state of SasA. 20 µL injections of full-length SasA at 1.5 mg/mL were made using an Agilent G1311A quaternary pump and manual injector (Rheodyne), run over the silica-based column, and analyzed by a T-rEX refractometer and miniDAWN TREOS II static multiangle light scattering instrument (Wyatt Technologies) directly after the column. Analysis of absolute molecular weight was carried out using Astra 6.0 software (Wyatt Technologies).

Generation of SasA mutants in *S. elongatus*

Markerless point mutations were introduced in *sasA* of *Synechococcus elongatus* PCC 7942 by CRISPR/Cas12a engineering as previously described.[152] Plasmids and the primers used in vector construction and sequence verification are listed in **Table 3.8**. Briefly, oligos with complementarity to the guide RNA (gRNA) recognition site were annealed and cloned into AarI-cut pSL2680 (Addgene Plasmid #85581). Clones of pSL2680 that carry the appropriate gRNA insert were isolated and plasmid sequences were verified. Upstream and downstream homologous repair templates that encode the point mutation(s) of interest were amplified by PCR and assembled (GeneArt Seamless Assembly, Thermo Fisher) into KpnI-cut constructs that contain the respective gRNAs. Recovered plasmids were checked for accuracy by Sanger sequencing prior to editing in *S. elongatus*.

The RSF1010-based editing constructs were electroporated into *E. coli* AM1359 that contain conjugal helper plasmids (pRL623 and pRL443) as previously described.[153–157] The resulting *E. coli* strains were grown overnight in LB containing ampicillin (100 µg/ml), chloramphenicol (17 µg/ml) and kanamycin (50 µg/ml). Cells from a 1 ml aliquot were washed three times with fresh LB and resuspended in a final volume of 100 µl LB, then mixed with 100 µl of an *S. elongatus* clock-reporter strain (AMC541) concentrated down from 2 ml of a dense culture (OD_{750} = ~0.6). The mixed culture was plated to solid BG-11 medium containing 5% LB (v/v) and incubated at 30˚C under 30 µmol photons m^{-2} s^{-1} (µE) illumination for 24 hours. Plates were then underlaid with kanamycin (5 µg/ml final concentration) to select for the editing plasmid. *S. elongatus* colonies

that emerged after 8-10 days at 30˚C and 100 µE light were serially patched three times to BG-11 containing kanamycin to maintain the editing plasmid long enough to complete segregation of the mutant allele in all copies of the chromosome. After editing, *sasA* was amplified by colony PCR using primers that anneal outside of the homologous repair region and the resulting PCR product was submitted for Sanger sequencing to confirm segregation of the point mutation(s) of interest.

Bioluminescence monitoring of *S. elongatus* circadian rhythms

The impact of *sasA* mutations on output from the KaiABC oscillator *in vivo* was measured using a firefly luciferase reporter driven by the *kaiBC* promoter (P_{kaiBC}::*luc*) as previously described.[158] Mutant *sasA* strains, along with positive and negative clock-output controls, were grown in BG-11 medium, diluted to OD_{750} = 0.2 and arrayed in 96-well plates containing solid BG-11 medium and 10 µl of 5 mM D-luciferin. Plates were covered by a gas permeable seal and incubated in a light-dark chamber at 30˚C for 48 hours, with 12 hour intervals of 120 µE light and darkness. Following release into constant light at the end of 48 hours, plates were transferred to a lighted stacker (40 µE light) attached to a Tecan Infinite M200 Pro and bioluminescence was monitored every 2-3 hours. The raw bioluminescence data were plotted as a function of time (GraphPad Prism 8) and processed using BioDare2 to determine period and amplitude for each set of replicates.[129] All strains used in this study are listed in **Table 3.9**.

Table 3.2. Constructs and shorthand names used in this study.

Shorthand name	organism	Protein full name
KaiC	*S. elongatus*	FLAG-seKaiC-1-518
KaiC-EE		FLAG-seKaiC-1-518-S431E-T432E
	T. elongatus	FLAG-teKaiC-1-517-S431E-T432E
KaiC-EA	*S. elongatus*	FLAG-seKaiC-1-518-S431E-T432A
KaiC-EE-D121A		FLAG-teKaiC-1-517-D121A-S431E-T432E
KaiC-EE-F122A		FLAG-teKaiC-1-517-F122A-S431E-T432E
KaiC-EE-D123A	*T. elongatus*	FLAG-teKaiC-1-517-D123A-S431E-T432E
KaiC-CI monomer		FLAG-teKaiC-17-247-R41A-K173A
		FLAG-seKaiC-16-246-R40A-K172A
KaiB		seKaiB-1-102-FLAG
KaiB-K25C-6IAF		sekaiB-1-102-K25C-FLAG-fluorescein
fsKaiB		seKaiB-1-99-Y7A-I87A-Y93A-FLAG
fsKaiB-R22A		seKaiB-1-99-Y7A-R22A-I87A-Y93A-FLAG
KaiA		seKaiA-1-284
SasA	*S. elongatus*	seSasA-1-387-FLAG
SasA-H161A		seSasA-1-387-H161A-FLAG
SasA-H28A		seSasA-1-387-H28A-FLAG
SasA-Q94A		seSasA-1-387-Q94A-FLAG
SasA-H28A-Q94A		seSasA-1-387-H28A-Q94A-FLAG
$SasA_{trx}$		FLAG-seSasA-13-103-P13A-FLAG
	T. elongatus	FLAG-teSasA-16-107-P16A-FLAG
$SasA_{trx}$-Q31C*	*S. elongatus*	FLAG-seSasA-13-103-P13A-Q31C-FLAG
$SasA_{trx}$-A35C*	*T. elongatus*	FLAG-teSasA-16-107-P16A-A35C-FLAG
CikA		FLAG-seCikA-1-754
$CikA_{psr}$	*S. elongatus*	seCikA-S605-606-745-C644S-C686S-C742S
$CikA_{psr}$-S727C*		seCikA-S605-606-745-C644S-C686S-C742S-S727C

* single cysteine residue for labeling with 6-iodoacetamido-fluorescein

Table 3.3. Specific reaction conditions with designated protein constructs.

Experiment Type	Protein Construct Shorthand Name / Final Concentration (μM if not indicated)	Experimental Condition
KaiC Phosphorylation Assay (Fig. 3.1B, 3.S1)	1) KaiC / 3.5 2) KaiB / 3.5 3) KaiA / 1.2	• Volume: 1.8 mL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
FP-PTO Assay tracking fluorescently labeled KaiB (Fig. 3.1B, 3.S1)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / 3.45 4) KaiA / 1.2	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
FP-PTO Assay tracking fluorescently labeled $SasA_{trx}$ (Fig. 3.1B, 3.S1)	1) $SasA_{trx}$-Q31C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / 3.5 4) KaiA / 1.2	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
KaiA Titration FP-PTO Assay (Fig. 3.4A)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / 3.45 4) KaiA / varied (0.3, 0.6, 1.2, 2.4, 3.6, 4.8, and 6.0)	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
KaiB Titration FP-PTO Assay (Fig. 3.4B)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / varied (0.65, 0.825, 1.70, 3.45, 6.95, 10.45, and 17.45) 4) KaiA / 1.2	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
SasA Titration FP-PTO Assay under different KaiB concentrations (Fig. 3.5)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / fixed at 0.825, 1.70, and 3.45 4) KaiA / 1.2 5) SasA / varied (0.0, 0.1, 0.65, 1.0, 1.8, 3.5, and 5.0)	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
CikA Titration FP-PTO Assay under different KaiA concentrations (3.S6)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / 3.45 4) KaiA / fixed at 0.3 and 1.2 5) CikA / varied (0.0, 0.1, 0.65, 1.0, 1.8, 3.5, and 5.0)	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C
$SasA_{trx}$ Titration FP-PTO Assay under different KaiB concentrations (3.S7A)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / fixed at 1.70 and 3.45 4) KaiA / 1.2 5) $SasA_{trx}$ / varied (0.0, 0.1, 0.65, 1.0, 1.8, 3.5, and 5.0)	• Volume: 80 μL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C

$CikA_{psr}$ Titration FP-PTO Assay under different KaiA concentrations (3.S7B)	1) KaiB-K25C-6IAF / 0.05 2) KaiC / 3.5 3) KaiB / 3.45 4) KaiA / fixed at 0.3 and 1.2 5) $CikA_{psr}$ / varied (0.0, 0.1, 0.65, 1.0, 1.8, 3.5, and 5.0)	• Volume: 80 µL • Buffer: 20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM EDTA, pH 8.0 • Temperature: 30 °C

Table 3.4. Period analysis, ordinary one-way ANOVA for FP-PTO assays comparing different oscillator conditions.

Protein-concentration (μM) comparison with / altered protein-concentration	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted *P* Value
KaiB-3.5 / KaiB-1.75	-1.6660	-3.238 to -0.093	Yes	*	0.0354
KaiB-3.5 / KaiB-7.0	0.5423	-1.394 to 2.479	No	ns	0.8847
KaiB-3.5 / KaiB-10.5	0.5118	-1.561 to 2.584	No	ns	0.9220
KaiB-3.5 / KaiB-17.5	1.2440	-0.692 to 3.181	No	ns	0.3047
SasA-0.0 / SasA-0.1	-0.4980	-1.678 to 0.682	No	ns	0.6220
SasA-0.0 / SasA-0.3	-1.0860	-2.266 to 0.094	No	ns	0.0758
SasA-0.0 / SasA-0.65	-2.5200	-3.700 to -1.340	Yes	***	0.0001
SasA-0.0 / SasA-1.0	-4.1180	-5.298 to -2.938	Yes	#	<0.0001
SasA-0.0 / SasA-1.8	-12.7600	-14.490 to -11.030	Yes	#	<0.0001
KaiB-1.75, SasA-0.0 / SasA-0.1	1.9530	0.529 to 3.377	Yes	**	0.0048
KaiB-1.75, SasA-0.0 / SasA-0.3	0.9688	-0.561 to 2.498	No	ns	0.3415
KaiB-1.75, SasA-0.0 / SasA-0.65	-0.1362	-1.666 to 1.393	No	ns	0.9997
KaiB-1.75, SasA-0.0 / SasA-1.0	-1.8610	-3.285 to -0.437	Yes	**	0.0074
KaiB-1.75, SasA-0.0 / SasA-1.8	-8.0090	-10.660 to -5.359	Yes	#	<0.0001
$SasA_{trx}$-0.0 / $SasA_{trx}$-0.1	-0.1257	-1.638 to 1.387	No	ns	0.9997

$SasA_{trx}$-0.0 / $SasA_{trx}$-0.3	-0.3682	-1.881 to 1.144	No	ns	0.9812
$SasA_{trx}$-0.0 / $SasA_{trx}$-0.65	-1.0080	-2.521 to 0.504	No	ns	0.3257
$SasA_{trx}$-0.0 / $SasA_{trx}$-1.0	-1.7710	-3.283 to -0.258	Yes	*	0.0160
$SasA_{trx}$-0.0 / $SasA_{trx}$-1.8	-3.6110	-5.123 to -2.098	Yes	#	<0.0001
$SasA_{trx}$-0.0 / $SasA_{trx}$-3.5	-7.3180	-8.831 to -5.806	Yes	#	<0.0001
$SasA_{trx}$-0.0 / $SasA_{trx}$-5.0	-8.3660	-10.030 to -6.701	Yes	#	<0.0001
KaiA-1.2 / KaiA-0.3	-5.4330	-6.896 to -3.969	Yes	#	<0.0001
KaiA-1.2 / KaiA-0.6	-1.2930	-2.833 to 0.247	No	ns	0.1285
KaiA-1.2 / KaiA-2.4	0.5891	-0.951 to 2.129	No	ns	0.8174
KaiA-1.2 / KaiA-3.6	0.9321	-0.717 to 2.581	No	ns	0.4766
KaiA-1.2 / KaiA-4.8	-1.5930	-3.702 to 0.516	No	ns	0.2017
KaiA-1.2 / KaiA-6.0	-4.1730	-6.985 to -1.361	Yes	**	0.0020
CikA-0.0 / CikA-0.1	-0.0469	-0.163 to 0.069	No	ns	0.8110
CikA-0.0 / CikA-0.3	-0.0372	-0.153 to 0.079	No	ns	0.9261
CikA-0.0 / CikA-0.65	-0.0067	-0.123 to 0.110	No	ns	0.9998
CikA-0.0 / CikA-1.0	0.0440	-0.072 to 0.160	No	ns	0.8500
CikA-0.0 / CikA-1.8	0.1113	-0.005 to 0.228	No	ns	0.0654
CikA-0.0 / CikA-3.5	0.1006	-0.016 to 0.217	No	ns	0.1145

CikA-0.0 / CikA-5.0	0.1906	0.074 to 0.307	Yes	***	0.0004
KaiA-0.3, CikA-0.0 / CikA-0.1	0.7500	-2.315 to 3.815	No	ns	0.9499
KaiA-0.3, CikA-0.0 / CikA-0.3	2.5450	-0.520 to 5.610	No	ns	0.1201
KaiA-0.3, CikA-0.0 / CikA-0.65	4.6930	1.895 to 7.492	Yes	**	0.0014
KaiA-0.3, CikA-0.0 / CikA-1.0	5.0200	2.222 to 7.818	Yes	***	0.0007
KaiA-0.3, CikA-0.0 / CikA-1.8	6.4400	3.642 to 9.238	Yes	#	<0.0001
KaiA-0.3, CikA-0.0 / CikA-3.5	7.4070	4.608 to 10.210	Yes	#	<0.0001
KaiA-0.3, CikA-0.0 / CikA-5.0	7.9230	5.125 to 10.720	Yes	#	<0.0001
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.1	3.0100	2.132 to 3.889	Yes	#	<0.0001
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.3	2.3610	1.386 to 3.336	Yes	#	<0.0001
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.65	1.6580	0.779 to 2.536	Yes	#	<0.0001
$CikA_{psr}$-0.0 / $CikA_{psr}$-1.0	0.8853	0.007 to 1.764	Yes	*	0.0476
$CikA_{psr}$-0.0 / $CikA_{psr}$-1.8	0.6953	-0.183 to 1.574	No	ns	0.1771
$CikA_{psr}$-0.0 / $CikA_{psr}$-3.5	0.5711	-0.404 to 1.546	No	ns	0.4758
$CikA_{psr}$-0.0 / $CikA_{psr}$-5.0	-0.1997	-1.078 to 0.679	No	ns	0.9893
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.1	2.2560	-1.984 to 6.496	No	ns	0.4607
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.3	1.9060	-2.334 to 6.146	No	ns	0.6179
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.65	1.1310	-3.109 to 5.371	No	ns	0.9285

KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-1.0	0.4160	-3.824 to 4.656	No	ns	0.9996
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-1.8	-3.6840	-7.924 to 0.556	No	ns	0.0974
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-3.5	-11.2400	-15.480 to -6.999	Yes	#	<0.0001
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.1	-0.0650	-6.407 to 6.277	No	ns	>0.9999
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.3	0.5050	-5.837 to 6.847	No	ns	0.9997
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.65	1.1300	-5.212 to 7.472	No	ns	0.9937
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-1.0	1.9050	-4.437 to 8.247	No	ns	0.9136
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-1.8	2.2100	-4.132 to 8.552	No	ns	0.8450
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-3.5	4.1100	-2.232 to 10.450	No	ns	0.3006
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-5.0	4.4700	-1.872 to 10.810	No	ns	0.2302

Ordinary one-way ANOVA performed with Dunnett's multiple comparisons test was used to compare period values of the FP-PTO under specified conditions: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. If not specified, FP-PTO assays were run with 1.2 μM KaiA, 3.45 μM KaiB, 3.5 μM KaiC, and 0.05 μM fluorescently-labeled KaiB probe.

Table 3.5. Amplitude analysis, ordinary one-way ANOVA for FP-PTO assays comparing different oscillator conditions.

Protein-concentration (μM) comparison with / altered protein-concentration	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted *P* Value
KaiB-3.5 / KaiB-1.75	0.0874	-0.0212 to 0.1961	No	ns	0.1437
KaiB-3.5 / KaiB-7.0	0.0421	-0.0742 to 0.1584	No	ns	0.7636
KaiB-3.5 / KaiB-10.5	0.0466	-0.0621 to 0.1552	No	ns	0.6484
KaiB-3.5 / KaiB-17.5	0.4340	0.3457 to 0.5222	Yes	#	<0.0001
SasA-0.0 / SasA-0.1	-0.0384	-0.1930 to 0.1162	No	ns	0.9098
SasA-0.0 / SasA-0.3	0.0223	-0.1323 to 0.1769	No	ns	0.9890
SasA-0.0 / SasA-0.65	0.1149	-0.0397 to 0.2694	No	ns	0.1799
SasA-0.0 / SasA-1.0	0.2204	0.0658 to 0.3750	Yes	**	0.0047
SasA-0.0 / SasA-1.8	0.4423	0.2160 to 0.6686	Yes	***	0.0003
KaiB-1.75, SasA-0.0 / SasA-0.1	-0.4206	-0.6723 to -0.1688	Yes	***	0.0007
KaiB-1.75, SasA-0.0 / SasA-0.3	-0.5336	-0.8040 to -0.2632	Yes	#	<0.0001
KaiB-1.75, SasA-0.0 / SasA-0.65	-0.5240	-0.7944 to -0.2535	Yes	***	0.0001
KaiB-1.75, SasA-0.0 / SasA-1.0	-0.3502	-0.6019 to -0.0984	Yes	**	0.0043
KaiB-1.75, SasA-0.0 / SasA-1.8	0.0076	-0.4608 to 0.4759	No	ns	>0.9999
$SasA_{trx}$-0.0 / $SasA_{trx}$-0.1	-0.0456	-0.1360 to 0.0449	No	ns	0.6219
$SasA_{trx}$-0.0 / $SasA_{trx}$-0.3	-0.0340	-0.1245 to 0.0564	No	ns	0.8555

$SasA_{trx}$-0.0 / $SasA_{trx}$-0.65	0.0643	-0.0262 to 0.1547	No	ns	0.2642
$SasA_{trx}$-0.0 / $SasA_{trx}$-1.0	0.1418	0.0514 to 0.2323	Yes	***	0.0009
$SasA_{trx}$-0.0 / $SasA_{trx}$-1.8	0.2437	0.1532 to 0.3341	Yes	#	<0.0001
$SasA_{trx}$-0.0 / $SasA_{trx}$-3.5	0.3859	0.2954 to 0.4763	Yes	#	<0.0001
$SasA_{trx}$-0.0 / $SasA_{trx}$-5.0	0.4243	0.3247 to 0.5239	Yes	#	<0.0001
KaiA-1.2 / KaiA-0.3	0.3983	0.1870 to 0.6096	Yes	***	0.0001
KaiA-1.2 / KaiA-0.6	0.1897	-0.0216 to 0.4010	No	ns	0.0919
KaiA-1.2 / KaiA-2.4	0.1200	-0.0913 to 0.3313	No	ns	0.4707
KaiA-1.2 / KaiA-3.6	0.2376	0.0115 to 0.4638	Yes	*	0.0365
KaiA-1.2 / KaiA-4.8	0.4218	0.1324 to 0.7111	Yes	**	0.0025
KaiA-1.2 / KaiA-6.0	0.4745	0.0887 to 0.8602	Yes	*	0.0115
CikA-0.0 / CikA-0.1	-0.0469	-0.1630 to 0.0693	No	ns	0.8110
CikA-0.0 / CikA-0.3	-0.0372	-0.1534 to 0.0790	No	ns	0.9261
CikA-0.0 / CikA-0.65	-0.0067	-0.1229 to 0.1095	No	ns	0.9998
CikA-0.0 / CikA-1.0	0.0440	-0.0722 to 0.1602	No	ns	0.8500
CikA-0.0 / CikA-1.8	0.1113	-0.0049 to 0.2275	No	ns	0.0654
CikA-0.0 / CikA-3.5	0.1006	-0.0156 to 0.2168	No	ns	0.1145
CikA-0.0 / CikA-5.0	0.1906	0.0744 to 0.3068	Yes	***	0.0004

KaiA-0.3, CikA-0.0 / CikA-0.1	-0.0983	-0.3229 to 0.1263	No	ns	0.7137
KaiA-0.3, CikA-0.0 / CikA-0.3	-0.1326	-0.3571 to 0.0920	No	ns	0.4194
KaiA-0.3, CikA-0.0 / CikA-0.65	-0.0559	-0.2519 to 0.1401	No	ns	0.9446
KaiA-0.3, CikA-0.0 / CikA-1.0	-0.1135	-0.3095 to 0.0825	No	ns	0.4393
KaiA-0.3, CikA-0.0 / CikA-1.8	-0.1124	-0.3085 to 0.0836	No	ns	0.4490
KaiA-0.3, CikA-0.0 / CikA-3.5	-0.1599	-0.3560 to 0.0361	No	ns	0.1412
KaiA-0.3, CikA-0.0 / CikA-5.0	-0.1116	-0.3076 to 0.0844	No	ns	0.4568
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.1	0.0118	-0.2952 to 0.3189	No	ns	0.9999
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.3	-0.0595	-0.3976 to 0.2786	No	ns	0.9971
$CikA_{psr}$-0.0 / $CikA_{psr}$-0.65	0.0200	-0.2870 to 0.3271	No	ns	0.9997
$CikA_{psr}$-0.0 / $CikA_{psr}$-1.0	0.0674	-0.2397 to 0.3744	No	ns	0.9899
$CikA_{psr}$-0.0 / $CikA_{psr}$-1.8	0.0870	-0.2201 to 0.3941	No	ns	0.9588
$CikA_{psr}$-0.0 / $CikA_{psr}$-3.5	0.1960	-0.1420 to 0.5341	No	ns	0.4727
$CikA_{psr}$-0.0 / $CikA_{psr}$-5.0	0.1054	-0.2016 to 0.4125	No	ns	0.9001
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.1	-0.0569	-0.4526 to 0.3388	No	ns	0.9957
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.3	-0.2672	-0.6629 to 0.1285	No	ns	0.2498
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-0.65	-0.3274	-0.7231 to 0.0683	No	ns	0.1199
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-1.0	-0.1683	-0.5640 to 0.2275	No	ns	0.6662

KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-1.8	-0.0890	-0.4848 to 0.3067	No	ns	0.9653
KaiB-1.75, $SasA_{trx}$-0.0 / $SasA_{trx}$-3.5	-0.0607	-0.4564 to 0.3351	No	ns	0.9946
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.1	-0.0648	-0.3795 to 0.2500	No	ns	0.9863
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.3	-0.0670	-0.3818 to 0.2477	No	ns	0.9833
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-0.65	-0.1085	-0.4233 to 0.2063	No	ns	0.8510
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-1.0	-0.0998	-0.4145 to 0.2150	No	ns	0.8920
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-1.8	-0.0593	-0.3740 to 0.2555	No	ns	0.9918
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-3.5	-0.1340	-0.4487 to 0.1808	No	ns	0.7026
KaiA-0.3, $CikA_{psr}$-0.0 / $CikA_{psr}$-5.0	-0.1477	-0.4625 to 0.1670	No	ns	0.6145

Ordinary one-way ANOVA performed with Dunnett's multiple comparisons test was used to compare amplitude values of the FP-PTO under specified conditions: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. If not specified, FP-PTO assays were run with 1.2 μM KaiA, 3.45 μM KaiB, 3.5 μM KaiC, and 0.05 μM fluorescently-labeled KaiB probe.

Table 3.6. Ordinary two-way ANOVA of period values from FP-PTO with pairwise comparisons showing how core oscillator components modulate SasA or CikA effects.

[Fixed protein-concentration] protein-concentration comparison with / altered protein-concentration (μM)	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	N1	N2
[SasA-0.0] KaiB-3.5 / KaiB-1.75	-1.666	-2.798 to -0.534	Yes	**	0.0021	7	12
[SasA-0.0] KaiB-3.5 / KaiB-7.0	0.542	-0.852 to 1.936	No	ns	0.6753	7	5
[SasA-0.0] KaiB-3.5 / KaiB-17.5	1.244	-0.150 to 2.638	No	ns	0.0907	7	5
[SasA-0.1] KaiB-3.5 / KaiB-1.75	0.020	-1.493 to 1.533	No	ns	>0.9999	5	5
[SasA-0.1] KaiB-3.5 / KaiB-7.0	0.808	-0.939 to 2.555	No	ns	0.5642	5	3
[SasA-0.1] KaiB-3.5 / KaiB-17.5	1.228	-0.519 to 2.975	No	ns	0.2316	5	3
[SasA-0.3] KaiB-3.5 / KaiB-1.75	-0.377	-1.983 to 1.230	No	ns	0.9059	5	4
[SasA-0.3] KaiB-3.5 / KaiB-7.0	0.826	-0.923 to 2.575	No	ns	0.5504	5	3
[SasA-0.3] KaiB-3.5 / KaiB-17.5	1.069	-0.679 to 2.818	No	ns	0.3404	5	3
[SasA-0.65] KaiB-3.5 / KaiB-1.75	-0.048	-1.654 to 1.559	No	ns	0.9997	5	4
[SasA-0.65] KaiB-3.5 / KaiB-7.0	0.560	-1.189 to 2.309	No	ns	0.7967	5	3
[SasA-0.65] KaiB-3.5 / KaiB-17.5	1.103	-0.645 to 2.852	No	ns	0.3152	5	3
[SasA-1.0] KaiB-3.5 / KaiB-1.75	-0.174	-1.687 to 1.339	No	ns	0.9868	5	5
[SasA-1.0] KaiB-3.5 / KaiB-7.0	1.041	-0.705 to 2.788	No	ns	0.3594	5	3
[SasA-1.0] KaiB-3.5 / KaiB-17.5	0.881	-0.865 to 2.628	No	ns	0.4959	5	3

[SasA-1.8] KaiB-3.5 / KaiB-1.75	2.000	-0.274 to 4.274	No	ns	0.0925	2	2
[SasA-1.8] KaiB-3.5 / KaiB-7.0	3.375	0.590 to 6.160	Yes	*	0.0149	2	1
[CikA-0.0] KaiA-1.2 / KaiA-0.3	-5.433	-6.313 to -4.552	Yes	#	<0.0001	7	6
[CikA-0.0] KaiA-1.2 / KaiA-0.6	-1.293	-2.220 to -0.366	Yes	**	0.0031	7	5
[CikA-0.0] KaiA-1.2 / KaiA-2.4	0.589	-0.338 to 1.516	No	ns	0.3347	7	5
[CikA-0.0] KaiA-1.2 / KaiA-3.6	0.932	-0.060 to 1.924	No	ns	0.0719	7	4
[CikA-0.1] KaiA-1.2 / KaiA-0.3	-4.394	-5.728 to -3.060	Yes	#	<0.0001	5	2
[CikA-0.1] KaiA-1.2 / KaiA-0.6	-1.441	-2.605 to -0.276	Yes	**	0.0098	5	3
[CikA-0.1] KaiA-1.2 / KaiA-2.4	0.449	-0.715 to 1.614	No	ns	0.7689	5	3
[CikA-0.1] KaiA-1.2 / KaiA-3.6	-0.174	-1.920 to 1.572	No	ns	0.9979	5	1
[CikA-0.3] KaiA-1.2 / KaiA-0.3	-2.871	-4.205 to -1.537	Yes	#	<0.0001	5	2
[CikA-0.3] KaiA-1.2 / KaiA-0.6	-1.113	-2.277 to 0.052	No	ns	0.0659	5	3
[CikA-0.3] KaiA-1.2 / KaiA-2.4	0.371	-0.794 to 1.535	No	ns	0.8669	5	3
[CikA-0.3] KaiA-1.2 / KaiA-3.6	0.274	-1.472 to 2.020	No	ns	0.9885	5	1
[CikA-0.65] KaiA-1.2 / KaiA-0.3	-1.215	-2.209 to -0.220	Yes	*	0.0120	5	3
[CikA-0.65] KaiA-1.2 / KaiA-0.6	-0.908	-1.902 to 0.086	No	ns	0.0824	5	3
[CikA-0.65] KaiA-1.2 / KaiA-2.4	0.222	-0.772 to 1.216	No	ns	0.9194	5	3
[CikA-1.0] KaiA-1.2 / KaiA-0.3	-1.308	-2.302 to -0.314	Yes	**	0.0061	5	3

[CikA-1.0] KaiA-1.2 / KaiA-0.6	-0.581	-1.576 to 0.413	No	ns	0.3817	5	3
[CikA-1.0] KaiA-1.2 / KaiA-2.4	0.055	-0.939 to 1.050	No	ns	0.9985	5	3
[CikA-1.8] KaiA-1.2 / KaiA-0.3	-0.550	-1.713 to 0.613	No	ns	0.6158	5	3
[CikA-1.8] KaiA-1.2 / KaiA-0.6	-0.870	-2.033 to 0.293	No	ns	0.2068	5	3
[CikA-1.8] KaiA-1.2 / KaiA-2.4	-0.047	-1.210 to 1.116	No	ns	0.9999	5	3
[CikA-1.8] KaiA-1.2 / KaiA-3.6	-1.510	-3.254 to 0.234	No	ns	0.1114	5	1
[CikA-3.5] KaiA-1.2 / KaiA-0.3	0.125	-0.870 to 1.119	No	ns	0.9839	5	3
[CikA-3.5] KaiA-1.2 / KaiA-0.6	-0.169	-1.163 to 0.826	No	ns	0.9620	5	3
[CikA-3.5] KaiA-1.2 / KaiA-2.4	0.321	-0.673 to 1.316	No	ns	0.7969	5	3
[CikA-5.0] KaiA-1.2 / KaiA-0.3	0.161	-1.002 to 1.324	No	ns	0.9926	5	3
[CikA-5.0] KaiA-1.2 / KaiA-0.6	-0.195	-1.358 to 0.968	No	ns	0.9847	5	3
[CikA-5.0] KaiA-1.2 / KaiA-2.4	-0.132	-1.295 to 1.031	No	ns	0.9965	5	3
[CikA-5.0] KaiA-1.2 / KaiA-3.6	-4.302	-6.046 to -2.558	Yes	#	<0.0001	5	1

Ordinary two-way ANOVA performed with Dunnett's multiple comparisons test was used to compare pairwise period values of the FP-PTO under specified conditions: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. Predicted least squared (LS) mean difference is reported instead of mean due to comparing means with different number of independent experiments. N1 and N2 specify the number of independent experiments that were able to be fit by FFT-NLLS with the online BioDare suite for the first and second conditions being compared, respectively.[128,129] If not specified, oscillators were run with 1.2 μM KaiA, 3.45 μM KaiB, 3.5 μM KaiC, and 0.05 μM fluorescently-labeled KaiB probe.

Table 3.7. Ordinary two-way ANOVA of amplitude values from FP-PTO with pairwise comparisons showing how core oscillator components modulate SasA or CikA effects.

[Fixed protein-concentration] protein-concentration comparison with / altered protein-concentration (μM)	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value	N1	N2
[SasA-0.0] KaiB-3.5 / KaiB-1.75	0.4340	0.3145 to 0.5535	Yes	#	<0.0001	7	12
[SasA-0.0] KaiB-3.5 / KaiB-7.0	0.0466	-0.1006 to 0.1937	No	ns	0.7909	7	5
[SasA-0.0] KaiB-3.5 / KaiB-17.5	0.0874	-0.0597 to 0.2346	No	ns	0.3543	7	5
[SasA-0.1] KaiB-3.5 / KaiB-1.75	0.0417	-0.1180 to 0.2014	No	ns	0.8745	5	5
[SasA-0.1] KaiB-3.5 / KaiB-7.0	0.0696	-0.1148 to 0.2540	No	ns	0.7056	5	3
[SasA-0.1] KaiB-3.5 / KaiB-17.5	0.1301	-0.0543 to 0.3145	No	ns	0.2293	5	3
[SasA-0.3] KaiB-3.5 / KaiB-1.75	-0.1321	-0.3016 to 0.0375	No	ns	0.1640	5	4
[SasA-0.3] KaiB-3.5 / KaiB-7.0	0.0437	-0.1409 to 0.2283	No	ns	0.9036	5	3
[SasA-0.3] KaiB-3.5 / KaiB-17.5	0.0752	-0.1094 to 0.2598	No	ns	0.6589	5	3
[SasA-0.65] KaiB-3.5 / KaiB-1.75	-0.2150	-0.3845 to -0.0454	Yes	**	0.0089	5	4
[SasA-0.65] KaiB-3.5 / KaiB-7.0	-0.0596	-0.2442 to 0.1250	No	ns	0.7932	5	3
[SasA-0.65] KaiB-3.5 / KaiB-17.5	0.0135	-0.1711 to 0.1981	No	ns	0.9966	5	3
[SasA-1.0] KaiB-3.5 / KaiB-1.75	-0.1468	-0.3065 to 0.0129	No	ns	0.0792	5	5
[SasA-1.0] KaiB-3.5 / KaiB-7.0	0.0456	-0.1388 to 0.2300	No	ns	0.8907	5	3
[SasA-1.0] KaiB-3.5 / KaiB-17.5	0.0321	-0.1523 to 0.2165	No	ns	0.9571	5	3

[SasA-1.8] KaiB-3.5 / KaiB-1.75	0.0185	-0.2246 to 0.2617	No	ns	0.9792	2	2
[SasA-1.8] KaiB-3.5 / KaiB-7.0	-0.0108	-0.3086 to 0.2869	No	ns	0.9952	2	1
[CikA-0.0] KaiA-1.2 / KaiA-0.3	0.3983	0.2520 to 0.5447	Yes	#	<0.0001	7	5
[CikA-0.0] KaiA-1.2 / KaiA-0.6	0.1897	0.0434 to 0.3361	Yes	**	0.0066	7	5
[CikA-0.0] KaiA-1.2 / KaiA-2.4	0.1200	-0.0264 to 0.2663	No	ns	0.1407	7	5
[CikA-0.0] KaiA-1.2 / KaiA-3.6	0.2376	0.0810 to 0.3943	Yes	**	0.0012	7	4
[CikA-0.1] KaiA-1.2 / KaiA-0.3	0.3469	0.1364 to 0.5574	Yes	***	0.0004	5	2
[CikA-0.1] KaiA-1.2 / KaiA-0.6	0.1197	-0.0641 to 0.3034	No	ns	0.3250	5	3
[CikA-0.1] KaiA-1.2 / KaiA-2.4	0.0546	-0.1291 to 0.2383	No	ns	0.8923	5	3
[CikA-0.1] KaiA-1.2 / KaiA-3.6	0.2974	0.0218 to 0.5730	Yes	*	0.0299	5	1
[CikA-0.3] KaiA-1.2 / KaiA-0.3	0.3029	0.0925 to 0.5134	Yes	**	0.0021	5	2
[CikA-0.3] KaiA-1.2 / KaiA-0.6	0.0862	-0.0976 to 0.2699	No	ns	0.6260	5	3
[CikA-0.3] KaiA-1.2 / KaiA-2.4	0.0688	-0.1150 to 0.2525	No	ns	0.7871	5	3
[CikA-0.3] KaiA-1.2 / KaiA-3.6	0.4703	0.1947 to 0.7459	Yes	***	0.0002	5	1
[CikA-0.65] KaiA-1.2 / KaiA-0.3	0.3491	0.1872 to 0.5111	Yes	#	<0.0001	5	3
[CikA-0.65] KaiA-1.2 / KaiA-0.6	0.0629	-0.0991 to 0.2248	No	ns	0.6955	5	3
[CikA-0.65] KaiA-1.2 / KaiA-2.4	0.0823	-0.0796 to 0.2443	No	ns	0.4976	5	3
[CikA-1.0] KaiA-1.2 / KaiA-0.3	0.2408	0.0788 to 0.4027	Yes	**	0.0017	5	3

[CikA-1.0] KaiA-1.2 / KaiA-0.6	0.0664	-0.0955 to 0.2284	No	ns	0.6594	5	3
[CikA-1.0] KaiA-1.2 / KaiA-2.4	0.0411	-0.1209 to 0.2031	No	ns	0.8874	5	3
[CikA-1.8] KaiA-1.2 / KaiA-0.3	0.1746	-0.0089to 0.3581	No	ns	0.0675	5	3
[CikA-1.8] KaiA-1.2 / KaiA-0.6	-0.0304	-0.2139 to 0.1531	No	ns	0.9854	5	3
[CikA-1.8] KaiA-1.2 / KaiA-2.4	0.0879	-0.0956 to 0.2714	No	ns	0.6045	5	3
[CikA-1.8] KaiA-1.2 / KaiA-3.6	0.2620	-0.0133 to 0.5373	No	ns	0.0673	5	1
[CikA-3.5] KaiA-1.2 / KaiA-0.3	0.1377	-0.0242 to 0.2997	No	ns	0.1154	5	3
[CikA-3.5] KaiA-1.2 / KaiA-0.6	-0.0361	-0.1980 to 0.1259	No	ns	0.9200	5	3
[CikA-3.5] KaiA-1.2 / KaiA-2.4	0.1386	-0.0234 to 0.3005	No	ns	0.1124	5	3
[CikA-5.0] KaiA-1.2 / KaiA-0.3	0.0961	-0.0874 to 0.2796	No	ns	0.5253	5	3
[CikA-5.0] KaiA-1.2 / KaiA-0.6	-0.0234	-0.2069 to 0.1602	No	ns	0.9947	5	3
[CikA-5.0] KaiA-1.2 / KaiA-2.4	0.0787	-0.1048 to 0.2622	No	ns	0.6934	5	3
[CikA-5.0] KaiA-1.2 / KaiA-3.6	0.2078	-0.0675 to 0.4830	No	ns	0.1999	5	1

Ordinary two-way ANOVA performed with Dunnett's multiple comparisons test was used to compare pairwise amplitude values of the FP-PTO under specified conditions: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.0001$. Predicted least squared (LS) mean difference is reported instead of mean due to comparing means with different number of independent experiments. N1 and N2 specify the number of independent experiments that were able to be fit by FFT-NLLS with the online BioDare suite for the first and second conditions being compared, respectively.[128,129] If not specified, oscillators were run with 1.2 μM KaiA, 3.45 μM KaiB, 3.5 μM KaiC, and 0.05 μM fluorescently-labeled KaiB probe.

Table 3.8. Plasmids and primers used in generating cyanobacterial strains.

Plasmids	Description	Source
pSL2680	CRISPR/Cas12a plasmid; Km resistance	Addgene (#85581)
pSL2680-HA	pSL2680 + SasA H28A substitution	This study
pSL2680-NA	pSL2680 + SasA N93A substitution	This study
pSL2680-NE	pSL2680 + SasA N93E substitution	This study
pSL2680-94	pSL2680 + SasA Q94A substitution	This study
pSL2680-HA/94	pSL2680 + SasA H28A and Q94A substitutions	This study
pSL2680-97	pSL2680 + SasA Q97A substitution	This study
pSL2680-QE	pSL2680 + SasA Q97E substitution	This study
pSL2680-101	pSL2680 + SasA Q101A substitution	This study
Primers	**Sequence (5'-3')**	
Primers used for pSL2680-HA		
H28A gRNA F	AGATTGCAGCGGGTTAAAAATATT	
H28A gRNA R	AGACAATATTTTTAACCCGCTGCA	
H28A homology arm upstream F	TAGCTTTAATGCGGTAGTTGGTACCATGATCGA CGCCTGTCGA	
H28A homology arm upstream R	TTTAACCCGCTGCACGATGGCCTGTGACAGGG GCCG	
H28A homology arm downstream F	CGGCCCCTGTCACAGGCCATCGTGCAGCGGG TTAAA	
Primers used for pSL2680-NA		
N93A gRNA F	AGATGCTAATTGATCGGTGAGGTC	
N93A gRNA R	AGACGACCTCACCGATCAATTAGC	
N93A homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA	
N93A homology arm upstream R	CTGGGGCAACTGGGCGGCTAATTGATCGGT	
N93A homology arm downstream F	ACCGATCAATTAGCCGCCCAGTTGCCCCAG	
N93A homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC	

Primers used for pSL2680-NE	
N93E gRNA F	AGATGCTAATTGATCGGTGAGGTC
N93E gRNA R	AGACGACCTCACCGATCAATTAGC
N93E homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
N93E homology arm upstream R	CTGGGGCAACTGCTCGGCTAATTGATCGGT
N93E homology arm downstream F	ACCGATCAATTAGCCGAGCAGTTGCCCCAG
N93E homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC
Primers used for pSL2680-94	
Q94A gRNA F	AGATGTTGGCTAATTGATCGGTGA
Q94AgRNA R	AGACTCACCGATCAATTAGCCAAC
Q94A homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
Q94A homology arm upstream R	CCACTGGGGCAACGCGTTGGCTAATTG
Q94A homology arm downstream F	CAATTAGCCAACGCGTTGCCCCAGTGG
Q94A homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC
Primers used for pSL2680 HA/94	
HA/94 gRNA F	AGATACGAAGAAAGCTCAGTGAGC
HA/94 gRNA R	AGACGCTCACTGAGCTTTCTTCGT
HA/94 homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
HA/94 homology arm upstream R	GCTCACTGAGCTTTCTTCGTGTATCCGCCAAAT TGT
HA94 homology arm downstream F	ACAATTTGGCGGATACACGAAGAAAGCTCAGT GAGC
HA94 homology arm downstream R	CAGATCCTCTAGAGTCGACGGTACC ATCGTGCCTGATCGAACA
Primers used for pSL2680-97	

Q97A gRNA F	AGATGGGCAACTGGTTGGCTAATT
Q97A gRNA R	AGACAATTAGCCAACCAGTTGCCC
Q97A homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
Q97A homology arm upstream R	CTGAACCAGCCACGCGGGCAACTGGTT
Q97A homology arm downstream F	AACCAGTTGCCCGCGTGGCTGGTTCAG
Q97A homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC
Primers used for pSL2680-QE	
Q97E gRNA F	AGATTGAGTGGCATCGACCTCACC
Q97E gRNA R	AGACGGTGAGGTCGATGCCACTCA
Q97E homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
Q97E homology arm upstream R	GGCTAATTGATCGGTGAGGTCGATGCCACTCA GCACTTGGC
Q97E homology arm downstream F	ACCTCACCGATCAATTAGCCAACCAGTTGCCC GAGTGGCTGG
Q97E homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC
Primers used for pSL2680-101	
Q101A gRNA F	AGATAACCAGCCACTGGGGCAACT
Q101A gRNA R	AGACAGTTGCCCCAGTGGCTGGTT
Q101A homology arm upstream F	CATTTTTTTGTCTAGCTTTAATGCGGTAGTTGG TACC CTGGCGATGGACTTGCACTCA
Q101A homology arm upstream R	AAAGGCCTCTTGCGCAACCAGCCACTG
Q101A homology arm downstream F	CAGTGGCTGGTTGCGCAAGAGGCCTTT
Q101A homology arm downstream R	GCCCGGATTACAGATCCTCTAGAGTCGACGGT ACC TTAGCAGGGCATGGTGTAGC
Primers used for colony PCR	
sasA1_SNP_chkF	CGAGTTAATGGGAGAGTCTCTGTC
sasA1_SNP_chkR	GGCCTAGCTCCGTGAACG

Table 3.9. Cyanobacterial strains used in this study.

Strain	Genotype (NS denotes neutral site)	Antibiotic resistance	Source
WT (AMC541)	NSII-P_{kaiBC}::*luc*	Cm	Lab collection
Δ*sasA* (AMC1192)	NSII-P_{kaiBC}::*luc* Δ*sasA*	Cm	Lab collection
sasA-H28A	NSII-P_{kaiBC}::*luc. sasA*-H28A	Cm	This study
sasA-N93A	NSII-P_{kaiBC}::*luc. sasA*-N93A	Cm	This study
sasA-N93E	NSII-P_{kaiBC}::*luc. sasA*-N93E	Cm	This study
sasA-Q94A	NSII-P_{kaiBC}::*luc sasA*-Q94A	Cm	This study
sasA-H28A/Q94A	NSII-P_{kaiBC}::*luc sasA*-H28A/Q94A	Cm	This study
sasA-Q97A	NSII-P_{kaiBC}::*luc. sasA*-Q97A	Cm	This study
sasA-Q97E	NSII-P_{kaiBC}::*luc. sasA*-Q97E	Cm	This study
sasA-Q101A	NSII-P_{kaiBC}::*luc sasA*-Q101A	Cm	This study

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Appendix A

Complete protein expression and purification protocol: including bacterial transformation, colony screening, expression of SUMO-FLAG-SeKaiC, cell harvesting and lysis, affinity chromatography purification, gel filtration chromatography product isolation, stirred cell protein concentrating, protein concentration determination via BSA standard curve assay, and protein quality check.

Example protocol printed here for protein *S. elongatus* KaiC with N-terminal FLAG tag. These protocols were prepared to be printed as individual notebooks for each protein being expressed in the LiWang Lab.

This protocol was derived from laboratory practices of YongGang Chang. Writing, formatting, and conception was done by Joel Heisler, Archana Chavan, and YongGang Chang.

Before you begin, read following instructions carefully.

1. Read the entire protocol and make sure you understand each and every step clearly.
2. Check availability of the equipment/instrument necessary for this experiment and reserve them well in advance.
3. Make sure you know how to operate each piece of equipment used in this protocol. If not, please get training from designated person before starting this protocol.
4. Check availability of materials required for the experiment. Make necessary buffers and solutions as described in protocol.
5. Follow each and every step in protocol exactly as described. Do not make adjustments/deviations at any stage.
6. Follow the time line in protocol precisely and only stop at indicated intermittent stopping points.

Green Boxes indicates the ONLY times during which the researcher may leave the lab area.

7. Note down clearly any deviations/variations introduced when following in this protocol. Make notes of every observation for future reference and troubleshooting purpose.

Date: _____ Day: _____

Day 1: Bacterial Transformation for Protein Expression (pg.# 1 of 2)

Time required for Day 1: (3 ½ hours)

Materials and Equipment:

1. BL21 (DE3) competent *E. coli* cells (from Stratagene/Agilent Cat# 200131). **Lot#: _____**
2. Plasmid DNA stock solution of 10 ng/µL. **Lot#: _____**
3. 500 µL of 2X-YT broth (aliquots frozen at -20ºC) **Lot#: _____**
4. LB-Agar-Kan+ plates. **Lot#: _____**
5. Glass spreader, spirit lamp, 42 ºC water bath, ice bucket, incubator-shaker, and 37 ºC incubator.

Preparation:

1. Fill the bucket with ice and take the competent cells out from 'New-cells box' in -80ºC freezer and place immediately on ice.
2. Get the plasmid DNA tube and keep that on ice, bring the ice bucket to your bench.
3. Let the cells thaw on ice for **15 minutes**. Check by inverting gently.
4. Turn on the **water bath** to maintain the temperature at **42 ºC**. Thaw the 2X-YT broth at room temperature.
5. Label an autoclaved 1.5 mL Eppendorf tube with the name of plasmid construct and chill on ice for **10 minutes**.

Procedure:

1. Aliquot **50 µL of BL21 (DE3) competent *E.coli* cells** to a pre-chilled eppendorf tube.
2. Invert the tube 2 times and then spin down the plasmid solution at **13,000 rpm for 30 seconds**.
3. Pipette **2 µL of plasmid** (10 ng/µL stock concentration) and add to the 50µL competent cells. Mix by pipetting up and down gently 2 times. Close the lid and **incubate the cells on ice for 30 minutes**.

(30 min on ice)

Start: _____ **End**: _____

Meanwhile, return the remaining competent cells to 'Used-cells box', and plasmid construct back to freezer

4. Transfer the eppendorf tube to a floating rack quickly. Place the floating rack in **42 ºC** water bath for **heat shock**, for **45 seconds**

Date: _____ Day: _____

Day 1: Bacterial Transformation for Protein Expression (pg.# 2 of 2)

1. Transfer the tube back to ice and **incubate for 2 minutes on ice**.
While cells are on ice, **warm the 2X-YT broth in 42 ºC** water bath.
2. Invert the **pre-warmed 2X-YT tube twice and then add 500 µL broth** to cells on ice. Close the lid, mix by gently inverting twice.
3. Incubate the cells in shaker at **37 ºC** and **220 rpm**, for **1 hour**.

(1 hour shaking)

Start: _____ **End**: _____

4. Get the cells from shaker and spin down at 5000 rpm for 2 minutes.
5. While cells are spinning, sterilize the bench top by spraying 70% ethanol and wipe clean with paper towel. Get the LB-Agar-Kan+ plate (kanamycin: 50 µg/mL) from 4 ºC fridge. Label the bottom of plate with name of plasmid, competent cells, date and name.
6. Ignite the spirit lamp and **sterilize the glass spreaders** by dipping in 70% alcohol and holding in flame for 3 seconds. Set the glass road aside to cool down (keep near the flame).
7. **From spun down ells, discard 450 µL** of supernatant and resuspend the cells in remaining 100 µL media by pipetting up and down gently.
8. **Transfer the cells** to the center of **agar** surface using a pipette. Get the pre-sterilized and cooled glass spreader and touch to the agar surface near edge of the plate to confirm that it is cool.
9. Spread the cells using spreader evenly across the entire surface of agar. Close the lid and keep the plate in the same position for **5 minutes on bench.**
10. **Sanitize** the **glass spreader** by dipping in 70 % alcohol contained in a beaker and then holding it in flame for 3 seconds.
11. Transfer the plate to the 37 ºC incubator and place it upside down (lid down). Incubate overnight (15 hours).

(15 hours incubation)

Start: _____ **End**: _____

12. Next day, count the number of colonies.

Number of colonies: _____

Perform colony screening for protein expression ***(see Protocol for Day-2)***.

Date: _____ Day: _____

Day 2: Colony Screening for Protein Expression (pg.# 1 of 2)

Time required for Day 2: (10 hours)

Materials and Equipment:

1. Bacterial transformation plate with colonies from Day-1. **Lot#:** _____
2. LB-Agar-Kan+ media. (50 mL Corning tubes at -20 ºC) **Lot#:** _____
3. 1 M IPTG stock (1 mL aliquots frozen at -20 ºC) **Lot#:** _____
4. 15 mL BD Falcon tubes, spirit lamp, incubator-shaker.

Preparation:

1. **Thaw** the **LB-Kan+ media** by placing the tube in D.I. water in 500 mL beaker.
2. **Label** 15 mL Falcon tubes as, #1, 2, 3, 4, 5 and 6.

Procedure:

1. Add **5 mL** of LB-Kan+ media into each of the 6 **Falcon tubes**.
2. Pick a **single colony**, using tip of 10 µL pipette and transfer to a LB-Kan+ media tube #1.

Pick 5 more colonies and transfer to LB-Kan+ media in tubes #2, #3, #4, #5 and #6.

3. Grow the cells by shaking at **220 rpm** and **37ºC** until OD600 = **0.5** units.

This step typically takes about **3 ½ to 4 hours** for OD600 to reach 0.5 units.

(3-4 hours Incubation)

Start: _____ **End**: _____

4. To measure OD600, take **500 µL of culture** from each tube and add **500µL of DI water**. Mix well and transfer to disposable plastic cuvettes. Measure OD600 on Hatch DR6000™ UV-Visible spectrometer.

Use **dilution factor of 2** to get **OD600** of original sample and record values below.

OD600 at (time) : _____

(1): _____ **(4):** _____

(2): _____ **(5):** _____

(3): _____ **(6):** _____

Follow **steps, 8-9 (during step-6)** to make **Pre-Induction Sample** for SDA-PAGE.

5. Take another 500 µL of culture in an autoclaved eppendorf tubes. During step 6, make **glycerol stocks** by following steps **10-12**.

Date: _____ Day: _____

Day 2: Colony Screening for Protein Expression (pg.# 2 of 2)

6. **Induce** remaining 4 mL of culture by adding 0.8 µL of 1M **IPTG** stock solution.

Induce at **30 ºC** with shaking, for **4 hours**.

(4 hour induction)

Start: _____ **End**: _____

7. After induction, take **500 µL of culture** and dilute with **500 µL DI water**. Measure **OD600** as described in **step-4**.

Use **dilution factor of 2** to get **OD600** of original sample and record values.

OD600 at (time): _____

(1): _____ **(4): _____**

(2): _____ **(5): _____**

(3): _____ **(6): _____**

Follow **steps 8 and 9** (after step-7),

Post-Induction Samples for SDS-PAGE

8. For making sample for SDS-PAGE, transfer the dilute culture from cuvettes to labeled eppendorf tubes (in step-4 and step -7). Spin down at **13,000 rpm for 2 minutes**.
9. **Discard the supernatant** and **resuspend** the cell pellet in **50 µL of 8 M urea** and **add 50 µL** of **2X-SDS-PAGE loading dye**. Save the tubes at room temperature. For further analysis see Protocols 3C and 3D.
10. For making glycerol stocks, spin down cells (immediately after step-5) at 5000 rpm, for 1 minute. Discard supernatant.
11. Resuspend cells in **400 µL of fresh LB-Kan+ media** and add **100 µL of 80 % glycerol** (autoclaved previously). Mix well by pipetting un-n-down 5 times.
12. **Label** each tube with details of plasmid, cells, antibiotic resistance, date, name and colony number. Place the tubes in 'Temporary glycerol stock' box and save at -80ºC.
13. After analyzing expression test results on SDS-PAGE (Day-3), save the good one and discard the glycerol stocks with lower protein expression.

Date: _____ Day: _____

Day 3: SDS-PAGE for Protein Expression Test (pg.# 1 of 2)

Time required for Day 3: (6 hours)

Materials and Equipment:

1. 17% SDS-PAGE gels (see Protocol #3 for making the gels). **Lot#: _____**
2. 1X Protein molecular size marker (BioRad 20X stock Cat #) **Lot#: _____**
3. 10X Glycine Buffer (see Protocol # 3) **Lot#: _____**
4. Electrophoresis buffer tank, electrode assembly, lid and power supply.

Preparation:

1. Assemble the SDS-PAGE gels and place them in buffer tank (see **Protocol 3)**.
2. Fill the space between plates with **1X glycine buffer 20 minutes** prior to running the gel, to **check for leakage**.

Procedure:

1. **Boil** the Pre-Induction and Post-Induction samples at **95 ºC**, for **5 minutes** in a heating block.
2. Spin down samples at **13,000 rpm for 1 minute**.
3. **Load 5 µL** (from left) of 1X protein **marker** in first lane.
4. For expression samples, calculate loading volume such that it gives an equivalent of **OD600** of **0.15** units for original **culture**.

Pair the pre- and post-induction samples for each colony number. Start loading in lanes from left to right, next to the protein marker.

5. **Fill the tank** with 1X glycine buffer, upto the mark indicating 2 gels.
6. Place the lid on the tank matching the electrodes. **Plug** in the other end of **electrodes to power supply**.
7. Run the following **electrophoresis program**.

Step 1: 60 V for 30 minutes

Step 2: 140 V for 1 hour, 40 minutes. (100 minutes).

8. After run, switch off the power supply and remove the cords and lid carefully.
9. Take gel-sandwich assembly out and empty gel tank by pouring buffer in sink.
10. Carefully remove the gel plates to separate the thin SDS-PAGE gel layer and place it in clean plastic Tupperware.
11. **Rinse** the gel **gently** using **D.I water** and pour the water out.
12. **Add Coommasie stain** to submerge the gel completely. Place the lid on loosely and **microwave** the gel in stain for **1 minute**.
13. Place the Tupperware on rotating shaker (in fume hood) for **30 minutes**.

Date: _____ Day: _____

Day 3: SDS-PAGE for Protein Expression Test (pg.# 2 of 2)

14. Drain out the stain into a 'Used-stain' bottle, while holding gel in position. Rinse the gel with D.I water.
15. Add '**Destaining solution'** to submerge the gel and keep the closed Tupperware on rotating shaker for **1 hour**.
16. Remove the destaining solution by draining into flask with charcoal filter.
17. **Repeat steps 15-16**. Place the gel in **D.I. water** and **visualize** against white background.
18. Compare the expression of fusion protein SUMO-FLAG-SeKaiC in pre- and post-induction samples for each colony (around molecular size **72 kDa**).
19. Sandwich the gel between hydrated cellophane. Keep it straight by clamping onto plastic plates using binder clips.
20. Let the gel **air dry** on gel plate rack (near window) for **1 day**.
21. Paste the dry gel below and mark the lanes for molecular sizes and sample names.

Protein expression test: SDS-PAGE gel:

At this stage, check the requirements for expression and purification of FLAG-SeKaiC in following protocol. The next steps for expression and purification need to be performed in a timely fashion, **so clear your schedule for next 4 days before starting.**

Date: _____ Day: _____

Day 1: Expression of SUMO-FLAG-SeKaiC (pg.# 1 of 3)

Time required for Day-1: (13 hours)

Materials and Equipment:

1. LB-Kan+ media. **Lot#:** _____
2. Glycerol stock (from SDS-PAGE analysis) **Lot#:** _____
3. Na2HPO4 **Lot#:** _____
4. KH2PO4 **Lot#:** _____
5. NH4Cl **Lot#:** _____
6. NaCl **Lot#:** _____
7. 20% D-Glucose **Lot#:** _____
8. 1 M MgSO4 **Lot#:** _____
9. 1 M CaCl2 **Lot#:** _____
10. 50 mg/mL Kanamycin **Lot#:** _____
11. 15 mL BD Falcon tubes – (number of tubes: 8)
12. 2 L culture flasks – (number of flasks: 8)
13. 2 L Beaker, 2 L graduated cylinder, magnetic stir plate, balance, shakers, autoclave, sterilization trays for autoclave, UV-Visible spectrometer.

Preparation:

1. **Thaw LB-Kan+ media** by placing the frozen tube in DI water (in 500 mL beaker).
2. Label each falcon tube with the name of protein construct being expressed.
3. Make **8 L of M9 salt solution** and **autoclave while starter culture is shaking at 37 ºC**.
4. **Thaw frozen stock solutions** of D-glucose, MgSO4, CaCl2 and Kanamycin **(30 minutes)** before transferring starter culture into **1 L of M9 media**.

Procedure-1: Starter Culture

1. **Sterilize bench** top using 70 % ethanol. Ignite spirit lamp and work near it.
2. Add **5 mL of LB-Kan+** media into each of eight, **Falcon tubes**.
3. Inoculate media in each falcon tubes by **adding 2 µL of glycerol stock**. (Use new pipette tip every time).
4. **Grow cells** in shaker (220 rpm) at **37 °C** for **6 ½ hours**.

(6 ½ hour induction)

Start: _____ **End:** _____

Make 8 L of M9 salt solution and autoclave while starter culture is shaking.

Date: _____ **Day:** _____

Day 1: Expression of SUMO-FLAG-SeKaiC (pg.# 2 of 3)

Making M9 Salt Solution

1. Obtain 2 L beaker and place large (1 ½") stirring bar inside.
2. Gather the following reagents for **8 L M9** media prep in a 2 L beaker.

8 L M9 Media Prep		Added
Na_2HPO_4 (Sodium Phosphate Dibasic Anhydrous)	**48.0 g**	**[ ]**
KH_2PO_4 (Potassium Phosphate Monobasic)	**24.0 g**	**[ ]**
NaCl (Sodium Chloride)	**4.0 g**	**[ ]**
NH_4Cl (Ammonium Chloride)	**8.0 g**	**[ ]**

3. Add all reagents to 2 L beaker with **1.5 L DI H2O**, and stir for **30 minutes**

Start: _____ **End**: _____

4. Use calibrated graduated cylinder to bring volume to 2 L.
5. Pour solution back and forth from beaker to cylinder three times to mix well.
6. Measure **500 mL M9 salt solution** and transfer to four of the 2 L flasks.
7. **Add 1.5 L DI H2O** to each of the four flasks with M9 salt solution.
8. Transfer the M9 solution back and forth from flask to graduated cylinder three times to mix.
9. Transfer 1 L of to each of the empty flasks until each flask has 1 L (1x) M9salt solution.
10. Cover flask mouth with **aluminum foil**. Place a small **strip of autoclave tape** on top.
11. Prepare autoclave trays with **1 ½ inch D.I. H2O**, and place flasks inside (3 per tray, 2 will sit on autoclave rack).
12. Autoclave M9 salt solution media on **Liquid Cycle #3 (121 °C, 15 psi, for 20 min)**. See Protocol # 5 for autoclave operation procedure.

Start: _____ **End**: _____

During autoclave cycle, thaw frozen stock solutions of (M9 media supplements), D-glucose, MgSO4, CaCl2 and Kanamycin.

13. Remove hot flasks from autoclave and **let cool to room temperature before adding M9 media supplements**.

Date: _____ Day: _____

Day 1: Expression of SUMO-FLAG-SeKaiC (pg.# 3 of 3)

Transfer Starter Culture and Induction:

1. **Add** following M9 media **supplements** to M9 salt solution before transferring the starter culture.

Add following solutions to each flask **(1 L M9 media)** and **mix by swirling the flask immediately after each addition**.

M9 Media Supplements to Each Flask	Added	
20 % D-Glucose	**10.0 mL**	**[ ]**
1 M MgSO4	**2.0 mL**	**[ ]**
1 M CaCl2	**100.0 µL**	**[ ]**
50 mg/mL Kanamycin	**1.0 mL**	**[ ]**

2. Transfer **5 mL of starter culture** to each flask, mix by swirling.
3. Place the flasks in shakers and incubate at **37 ºC** with shaking at **220 rpm**.

Let cells grow until **OD600 of 0.5**

Typically takes (6 hours), check OD600 at 5 ½ hours after transfer.

OD600 at (time): _____

(1): _____ **(5): _____**

(2): _____ **(6): _____**

(3): _____ **(7): _____**

(4): _____ **(8): _____**

4. Induce protein expression by adding **200 µL** of **1 M IPTG** into each flask.

Change the shaker temperature to **30 ºC** and **induce cells** for **12 hours**.

(12 hours Induction at 30 ºC)

Start: _____ **Stop: _____**

5. **Stop induction** at 12 hours. Transfer culture flasks from shaker to cart and follow **procedures for Day 2**.

While cultures are growing for 5-6 hours (in step 3), clean and regenerate four Ni-NTA columns, check availability of buffers for Ni-NTA column purification and make new buffers if necessary. (See protocol #6 and 10).

Date: _____ Day: _____

Day 2: Harvesting Cells for Purification SUMO-FLAG-SeKaiC (pg.# 1 of 4)

Time required for Day-2: (7 hours)

Materials and Equipment:

1. Lysis Buffer. **Lot #:** _____

50 mM NaH2PO4, 500 mM NaCl, 1 mM ATP, 1 mM MgCl2, pH 8.0

2. Sorval RC6 high-speed centrifuge, FS-10 rotor and lid, centrifuge bottles with lids.
3. Weighing balance, plastic try with ice, disposable serological pipette and bulb.

Procedure:

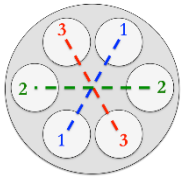
1. Place **FS-10 rotor** onto the spindle carefully, without tilting. Turn the **main switch on**.
2. Check the correct sizes of all the centrifuge bottles. Examine bottle for cracks or broken lids. **Do not use cracked bottles or lids.**
3. Pour the cell culture up to **3/4th height** in each of the 6 bottles (roughly 350 mL) and balance bottles with their matching lids**, within ±0.05 g difference**. (Use D.I. water to adjust small weight difference).
4. Dry the outside of the bottles with paper towel and place them in the **FS-10 rotor** in a balanced manner (see figure below).
5. Screw the rotor lid on and tighten it for securing the rotor in place. Test security of the rotor by lifting from bottom. A secured rotor allows no movement.

Lid ON [ ] Rotor Secured [ ]

6. Close centrifuge door and set the following parameters on display panel

Rotor FS-10 (rotor code = 47) [ ] Speed = 5000 rpm [ ]

Time = 8 minutes [ ] Temperature = 4 ºC [ ]



7. Press the **'Start'** button. Stay and observe until the desired speed is reached. Make sure no noise or vibration is detected.

If abnormal noise or vibration is detected, press the 'Stop' button immediately and recheck everything.

8. An alarm will ring when the centrifugation cycle is complete. Let the rotor make complete stop before opening the centrifuge door.
9. Loosen rotor lid and keep the lid (upside down) on a bench. Take centrifuge bottles out, and place on a lab cart. **Discard the supernatant** in the sink.
10. **Repeat step 3-8**, until all the cells are spun down. Keep the **cell pellet** on **ice**.
11. Resuspend the cells from each centrifuge bottle in **40 mL of lysis buffer**. **Combine** the resuspended cells (**240 mL**) in a clean beaker and keep on ice for cell lysis for cell lysis. ***(See homogenizer protocol)***.

Date: _____ Day: _____

Day 2: Cell Lysis, Avestine C3 Emulsiflex Homogenizer (pg.# 2 of 4)

Materials and Equipment:

1. Avestine C3 Emulsiflex high-pressure homogenizer and nitrogen tank.
2. Submersible water pump, plastic tray with icy water.
3. Chilled DI water, hot water for cleaning, 5 % Contrad 70 detergent, 50 % ethanol.

Procedure:

1. **Switch ON** the homogenizer and open the nitrogen pressure regulator knob.
2. Turn **red stop knob clockwise** and then **push green button** to pump residual ethanol out. **Rinse** by **adding 50 mL DI water** and flush it out.
3. Add **30 mL of 5% Contrad 70 soap solution,** and hold outlet tube in sample cylinder to circulate the solution. When the outlet tube is free of bubbles, apply pressure by turning the air regulator **(gray) knob, clockwise**. Adjust air pressure to 70-80 psi so that big gauge working pressure reads between 15,000 to 20,000 psi.
4. **Circulate soap** solution from outlet tube to the sample cylinder for one minute at 15,000 psi. Lower the pressure and let soap solution flow out to waste container.
5. **Repeat steps 3 and 4** twice using **200 mL of warm water** each time, followed by 200 mL DI water. **Press red button** to **stop** the homogenizer.
6. Remove the sample cylinder and **chill on ice for 10 minutes**.
7. Fill the plastic tray with icy water. Start circulating **chilled water** through 'Heat Exchanger' by plugging in the pump (placed in icy water) to a power outlet.
8. **Rinse** sample cylinder with **DI water** and install back to the top.
9. **Load your samples** into sample cylinder and direct the outlet tube back to the cylinder. **Turn on the homogenizer**.
10. Let the samples run through the tube back to the cylinder and make sure the flow is continuous and there are no bubbles in tube.
11. Apply **15,000 to 20,000 psi** pressure to **lyse** the cells. **Circulate** cells for **8 minutes** then transfer tube outlet to a clean 250 mL beaker chilled on ice.
12. **Repeat steps 10 and 11** for second pass of sample through homogenizer.
13. Lower the **pressure to zero** and collect residual sample in a beaker.
14. Transfer outlet tube to waste container and **fill sample cylinder with hot water**. Let **rinse for 2 minute** and press **stop button**.

Start spinning the lysate in centrifuge then come back to clean the homogenizer.

Date: _____ Day: _____

Day 2: Centrifuge Cell Lysate using SS34 Rotor (pg.# 3 of 4)

Materials and Equipment:

1. Sorval RC6 high-speed centrifuge, SS34 rotor and lid.
2. Weighing balance, plastic tray with ice.

Turn the centrifuge on before starting cell lysis to cool it to 4 ºC.

Procedure:

1. Place **SS34 rotor** onto the spindle carefully, without tilting.
2. Check the correct sizes of all the centrifuge bottles. Examine bottle for cracks or broken lids. **Do not use cracked bottles or lids.**
3. Pour the cell lysate up to **3/4th height** in each of the 8 bottles (roughly 30 mL) and balance bottles with their matching lids, within **±0.05 g difference**. (**Use lysis buffer to adjust weight difference**).
4. Dry the outside of the bottles with paper towel and place them in SS34 rotor.
5. Screw the rotor lid on and tighten it for securing the rotor in place. Test security of the rotor by lifting from bottom. A secured rotor allows no movement.

Lid ON [ ] Rotor Secured [ ]

6. Close centrifuge door and set the following parameters on display panel

Rotor SS34 (rotor code = 5) [ ] Speed = 15, 000 rpm [ ]

Time = 45 minutes [ ] Temperature = 4 ºC [ ]

7. Press the **'Start'** button. Stay and observe until the desired speed is reached. Make sure no noise or vibration is detected.

If abnormal noise or vibration is detected, press the 'Stop' button immediately and recheck everything.

During centrifuge cycle, clean the homogenizer. (Follow steps 3-5 to clean homogenizer and add 100 mL of 50 % ethanol to fill the outlet tube and sample cylinder and close the lid and stop homogenizer). Equilibrate four, Ni-NTA columns.

8. An alarm will ring when centrifugation cycle is complete. Let the rotor make complete stop before opening the centrifuge lid.
9. Loosen rotor lid and keep the lid (upside down) on bench. Take centrifuge bottles out, and keep in the wire rack.
10. Switch the **centrifuge off**, **clean the chamber** and rotor and keep on rotor and lid on bench properly.
11. On your bench, collect the supernatant from all 8 tubes, in to a 250 mL beaker.

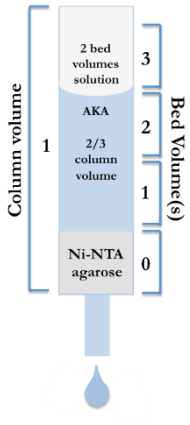
Date: _____ **Day:** _____

Day 2: Affinity Chromatography, Ni-NTA Gravity Column (pg.# 4 of 4)

Transfer the supernatant to eight, 50 mL Corning tubes **(30 mL each)** and bring tubes to 4 ºC fridge for affinity column purification on Ni-NTA columns.

Materials and Equipment:

1. Four, Ni-NTA gravity columns.
2. Lysis buffer. **Lot #:** _____
   50 mM NaH_2PO_4, 500 mM NaCl, 1 mM ATP, 1 mM $MgCl_2$, pH 8.0.
3. Wash buffer. **Lot #:** _____
   50 mM NaH_2PO_4, 500 mM NaCl, 80 mM imidazole, 1 mM ATP, 1 mM $MgCl_2$, pH 8.0
4. Elution buffer. **Lot #:** _____
   50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, 1 mM ATP, 1 mM $MgCl_2$, pH 8.0
5. 100 µM ULP-1 stock solution. **Lot #:** _____
6. 2X-SDS-PAGE loading buffer. **Lot #:** _____



Procedure:

1. Equilibrate **Ni-NTA columns** by running **2 column volumes of DI H_2O** followed by **1 column volume of Lysis buffer**.
2. Stop flow of Lysis buffer at 1 bed volume and clamp until use **(place in 4 °C)**.
3. Collect a **20 µL sample** of the **supernatant** before running on the column in a 1.5 mL centrifuge tube. **Load supernatant,** 60 mL onto each column without disturbing beads. Use disposable pipette to gently transfer the supernatant along wall of column.
4. Collect 20 **µL of flow-through** in separate tube. Mix each of the collected fractions with 20 µL of 2X-SDS-PAGE loading buffer.
5. Wash columns with **50 mL of Wash Buffer**, addition by disposable pipet applied to walls of column. Collect a **20 µL** sample of the **wash** (flow through) and mix with 20 µL of 2X-SDS-PAGE loading buffer.
6. Setup clean, labeled 50 mL Corning tubes under each Ni-NTA column in preparation for collected SUMO-FLAG-SeKaiC elution.
7. Elute SUMO-FLAG-SeKaiC by adding 6 mL of Elution Buffer to each column. Collect a 20 µL sample of the elution and mix with 20 µL 2X-SDS loading buffer.
8. Add **200 µL ULP-1** to each sample, mix by pipetting up-n-down 5 times and **cleave at 4 ºC overnight**.

(15 hours cleavage at 4 ºC)

Start: _____ **Stop:** _____

Date: _____ Day: _____

Day 3: Gel Filtration Chromatography (pg.# 1 of 5)

Time required for Day-3: (9 hours)

Materials and Equipment:

1. Tris base. **Lot #:** _____
2. Sodium Chloride (NaCl). **Lot #:** _____
3. Magnesium Chloride (MgCl2). **Lot #:** _____
4. ATP. **Lot #:** _____
5. EDTA. **Lot #:** _____
6. 1 L and 250 mL beakers, 1 L graduated cylinder, weighing balance, 1 L filter flask, vacuum pump, degassing adaptor, sonicator, spray bottle, 0.2 µm membrane filter.

Make Gel Filtration Buffer and Equilibrate the Column:

1. Prepare 1 L, Fixed Pressure Liquid Chromatography (FPLC) buffer in 1 L beaker:

Reagent	Weight	Added	
Tris-base (20 mM)	**2.42 g**		[ ]
NaCl (150 mM)	**8.77 g**		[ ]
$MgCl_2$ (5 mM)	**1.02 g**	"weighed on paper"	[ ]
ATP (1 mM)	**0.61 g**	"weighed on paper"	[ ]
EDTA (0.5 mM)	**0.21 g**	"weighed on paper"	[ ]

2. Dissolve reagents in **900 mL DI H_2O** on stirring plate, stirring bar **spinning slowly**.
3. Adjust solution to **pH 8.0** with bench-top pH meter using **6 M HCl**.
4. Transfer pH corrected solution to 1 L Graduated cylinder (marked **"Calib 1"**) and **bring volume to 1 L** (handwritten mark) with **DI H_2O**.

Use an additional 250 mL beaker to transfer water smoothly to the graduated cylinder.

5. Filter solution through **0.2 µm membrane** filter (GE nylon membrane), using **vacuum pump** and **filtering flask**.
6. Transfer filtered buffer into 1 L dry autoclaved bottle and label with reagents, pH, date, initials, filter, and degassed.
7. Degas buffer for **20 minutes** in **sonicator**, attached to vacuum pump via degassing adapter.

During degassing, fill two 250 mL beakers with DI H2O and use to PumpWashBasic the FPLC pump A.

8. Use clean **DI H2O spray bottles** to **rinse** pump between washes.
9. Before column equilibration, run three PumpWashBasic cycles. Each time, use separate beaker with DI water.

Date: _____ Day: _____

Day 3: Gel Filtration Chromatography (pg.# 2 of 5)

Materials and Equipment:

1. FPLC buffer:
20 mM Tris, 150 mM NaCl, 1 mM MgCl2, 1 mM ATP, 0.5 mM EDTA, pH 8.0.
2. Superdex 200 Hi-Load 1660- prep-grade column (GE Healthcare Cat#).
3. FPLC Instrument, FPLC injection adaptor.
4. 10 mL disposable syringes, injection needle, 100 mL beaker.
5. Tabletop centrifuge, 1.5 mL centrifuge tubes, large tweezers.
6. DI water spray bottles, 50 mL Corning tubes and rack, 500 mL beaker.

Equilibration and Gel Filtration on FPLC Column.

1. Obtain filtered and degassed FPLC buffer and setup new FPLC buffer as the running buffer (pump A), after **three PumpWashBasic cycles** in DI H2O with two, 250 mL beakers. (Change water for each PumpWashBasic cycle).
   Use DI H2O spray bottle to rinse the pump between washes.
2. Transfer pump A to degassed FPLC buffer and run one additional PumpWashBasic cycle.
3. In the FPLC software, setup the following parameters.

Column Volume:	**123 mL**
Max Pressure Limit:	**0.5 MPa**
Pump Flow:	**0.8 mL/min**
End Time:	**160 mL**

4. During equilibration, **concentrate cleaved samples to 12 mL** in preparation for **2 runs** on FPLC ***(See concentration protocol)***. Once samples are concentrated, aliquot the concentrated samples into eight **1.5 mL** centrifuge tubes (1.5 mL in each tube) and keep in 4 ºC fridge until next step.
5. After **Superdex 200 column** is equilibrated, spin down four sample tubes at **13,000 rpm** for **2 minutes** to pellet any aggregated proteins and/or particles and transfer supernatant into 100 mL beaker (make sure not to transfer any pellet).
6. Connect **injection needle** to a new **10 mL syringe** and **collect** the **supernatant** from beaker.
7. Exchange injection needle for FPLC adapter and **remove all bubbles** by tapping syringe with tweezers and **load sample** into **5 mL injection loop** on FPLC system.
   Use DI H2O spray bottle to ensure injection valve is full of water as the sample is loaded (avoiding any air bubbles being loaded onto column).

Date: _____ Day: _____

Day 3: Gel Filtration Chromatography (pg.# 3 of 5)

8. In the **[System] tab** select **(Run)** and find program with the following parameters.

Column:	Superdex200 Hiload1660 Prep-grade
Max Pressure:	0.5 MPa
Flow Rate:	0.8 mL/min
Empty Loop with:	**10 mL**
Outlet Valve:	**F3**
Run Volume:	**1.1 column volumes**

9. Start program and setup tube rack and pre-labeled 50 mL corning tubes.

Repeat steps 5-10 for second run to purify remaining cleaved sample.

Fraction Collection on Gel Filtration Chromatography.

1. Keep **outlet tube (on F3 valve)** in **clean 500 mL beaker** for entire run.
2. Collect FLAG-SeKaiC **elution at 56-64 mL**, in a clean **50 mL Corning tube**.
3. Keep the collected fraction from first run in **4 ºC** until fraction from second run is collected.
4. Save **20 µL aliquot** and mixed with **20 µL 2X-SDS-PAGE loading buffer**.
5. **Combine fractions** from both the FPLC runs and proceed to concentration step. ***(See concentrator protocol below)***.
6. **Concentrate** the combined gel filtration fractions for FLAG-SeKaiC to final volume of roughly, **1 mL**.
7. Collect the concentrated fraction in 1.5 mL centrifuge tube, and **spin down** at **13,000 rpm** for **2 minutes, at room temperature**.
8. **Aliquot** supernatant in 5 tubes, **200 µL each**.

Pre-label the tubes with the protein name, buffer, pH, date of preparation and Lot number.

> Lot#: [Preparor's initials]-[date]YYYYMMDD-[ID]
> Example: AC-20171023-SeC

9. Save **20 µL** supernatant in separate tube for concentration measurement. ***(See Bradford assay protocol)***.
10. Place all the **protein stocks** in a labeled **freezer box** and store in **-80 ºC freezer**.

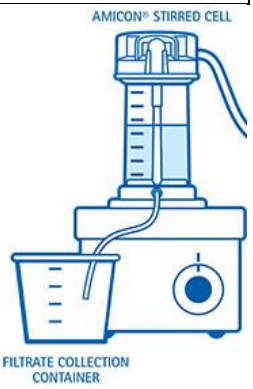
Date: _____ Day: _____

Day 3: Protein Concentration, Amicon Stirred Cell (pg.# 4 of 5)

Material and Equipment:

1. 50 mL stirred cell concentrator assembly.
2. 10 kDa Molecular weight cut off (MWCO) membrane, 45 mm diameter.
3. Flowthrough collection bottle, clean 100 mL beakers (2).
4. Coomassie dye. (Cat#)
5. N2 gas tank.
6. 8 M Urea.
7. Protein sample to be concentrated.

Concentrate Cleaved Protein After Ni-Column (Day-2)



1. Obtain cleaved protein sample following Ni-NTA column purification.
2. **Rinse** 50 mL concentrator with **DI H2O** thoroughly. Replace the old membrane with a new membrane.
3. Wash thoroughly with DI H2O at the sink (**fill 2/3 way** with water, **swirl** and **dump**, **25 times**).
4. Fill the concentrator with **30 mL DI H2O** and assemble the top.
5. Place the concentrator inside the black cage adapter and sit on stirring plate inside the **4 °C refrigerator**.
6. **Connect** the **N2 gas line** to the top of the concentrator. Ensure that the N2 gas line cutoff valve is in the closed position inside the refrigerator.
7. Place a temporary plastic bottle in front of concentrator to **collect flow through** (filtrate collection container).
8. **Open** the **gas** on the nitrogen tank by twisting the top valve to the left, and then **open** the **secondary valve** on the tank.
9. For the final gas line cutoff valve inside the refrigerator, ensure that the **lid** of the concentrator is **held in place** while **slowly opening the valve** (this is to prevent the gas from flooding into the concentrator rapidly and pushing the lid quickly up into the black cage adapter and possibly cracking it).
10. Turn the stirring plate to the **lowest speed** and allow **2 minutes** of water flow through the membrane (all 20 mL water to flow through the membrane).
11. **Disconnect** concentrator from **gas line** by **closing the cutoff valve** (inside the refrigerator) and **empty remaining water**.
12. **Rinse** the **concentrator** cells with the **same buffer** as that of protein sample **before adding protein sample**.
13. Carefully **pour cleaved protein sample** into the **empty concentrator**.
14. Place concentrator, in black adapter cage, onto of the stirring plate, and **connect the N2 gas line**.

Date: _____ Day: _____

Day 3: Protein Concentration, Amicon Stirred Cell (pg.# 5 of 5)

15. Place a 100 mL flask, labeled with protein being concentrated, in front of the concentrator to collect flow through.
16. **Open** the **gas** line cutoff valve and turn the stirring rate to the lowest setting.
17. During concentrating, sample **20 µL** of the **flow through** and mix with **20 µL of Coomassie dye** (compare to 20 µL buffer with dye and if flow through turns more blue, protein is leaking).

If protein is leaking, exchange membrane for a new hydrated (10 minutes in DI H2O) 10 kDa MWCO membrane.

18. **Concentrate** cleaved protein sample to **12 mL** for **2 runs on FPLC**.

Follow FPLC Gel Filtration run protocol after this step.

19. When finished, **close N2 gas cutoff valve**, and **turn off stirring plate**.
20. **Shut gas valves** on N2 tank to avoid slow leak while not in use.

N2 tank valves closed [ ] Pre-FPLC [ ] Post-FPLC [ ]

Concentrate Protein After Gel Filtration (Day-3)

After collecting fractions from both FPLC run for gel filtration chromatography of FLAG-SeKaiC, follow concentration protocol above and proceed to following steps.

1. Follow steps 12-20 above (concentration protocol, page #34 and 35).
2. **Concentrate combined protein sample to roughly 1 mL.**
3. Collect the concentrated fraction in 1.5 mL centrifuge tube, and **spin down** at **13,000 rpm** for **2 minutes, at room temperature**.
4. **Aliquot** supernatant in 5 tubes, **200 µL each**.
5. Pre-label the tubes with the protein name, buffer, pH, date of preparation and Lot number.
6. Save 20 µL supernatant in separate tube for concentration measurement. (See Bradford assay protocol).
7. Place all the protein stocks in a labeled freezer box and store in -80 ºC freezer.

Follow Bradford Assay Protocol for concentration measurement (Day-4).

8. Rinse concentrator with DI H2O a 10 times.
9. Add 8 M Urea to submerge the membrane, and manually stir/swirl for 20 seconds.
10. Wash thoroughly with DI H2O at the sink (fill 2/3 way with water, swirl and dump, 25 times).
11. Repeat steps #3-11 in concentrator protocol to fully wash out any residual urea from the membrane.
12. Repeat steps #19 and #20, and then store concentrator in 4 °C refrigerator.

Date: _____ Day: _____

Day 4: Measure Protein Concentration, Bradford Assay (pg.# 1 of 4)

Time required for Day-4: (9 hours)

Material and Equipment:

1. Concentrated protein sample. **Lot#:** _____
2. Bovine Serum Albumine (BSA) 2 mg/mL stock. (ThermoScientific Cat#)
3. Coomasssie Plus Bradford Assay Reagent, (ThermoScientific Cat# 23236)
4. NanoDrop™ 2000 UV-Visible spectrometer.

Procedure: Standard Curve Measurement

1. Acquire protein samples from concentrator protocol.
2. Prepare 200 µL BSA Standards of following concentrations (using in 0.9% NaCl, 0.02% NaN_3 buffer for dilution).

0 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL

Store labeled BSA Standards at 4 ºC for 2 months and make new standards after that. BSA Standards **Lot#:** _____

3. In pre-labeled 1.5 mL centrifuge tubes, **aliquot 150 µL** of **Coomassie dye** (room temperature).
4. Start **adding 5 µL** of **BSA standard** to each tube (corresponding label), **at 1 min interval** between each standard addition. Mix by pipetting up and down 3 times and invert the tube twice.

Start timer after adding the first standard and monitor time interval of 1 min for each subsequent standard additions.

5. **Incubate** each sample at room temperature for **10 minutes**. Invert the tube twice before each measurement on NanoDrop.
6. Use **5 µL** of **DI H_2O** for **blank** measurement.
7. Record absorbance at **595nm** for each standard and record values below.

BSA	A595 (1)	A595 (2)	A595 (3)
0 µg/mL			
200 µg/mL			
400 µg/mL			
600 µg/mL			
800 µg/mL			

8. Determine **linear fit** and attach standard curve with linear fit equation and R^2.

Date: _____ Day: _____

Day 4: Measure Protein Concentration, Bradford Assay (pg.# 2 of 4)

Procedure: Sample Measurements

Use standard curve in the format: $y = mx + b$

y = absorbance at 595nm

x = concentration in µg/mL

m = slope of curve

b = constant (*y-intercept*)

9. Prepare protein sample **dilutions** such that when adding **5 µL dilute protein** to **150 µL Coomassie dye** such that the blue coloration falls **within** the **standard range**.
10. Make **three identical samples** of FLAG-SeKaiC for measurement and record the absorbance for each sample below. (Set 1).
11. Repeat step 10 for another triplicate (set 2)of sample and record the absorbance below.

Sample	Set (1)	Set (2)
A595(1)		
A595(2)		
A595(3)		
Concentration		

12. **Calculate protein concentration** using following equation for each set of triplicate separately (record values in table

$$\frac{\left(\frac{y-b}{m}\right) \times \textit{Dilution Factor}}{\textit{Protein Molecular Weight (kDa)}} = \_\_\_\_\_ \ \mu M$$

Average Concentration of FLAG-SeKaiC: _____ µM

Lot #_____

Proceed to quality control UV scan for concentrated protein faction and attach the results.
Run SDS-PAGE aliquots of protein, saved during purification stages on 17 % SDS-PAGE gel. (See SDS-PAGE and silver stain protocols).

Attach the gel picture and analysis.

Date: _____ Day: _____

Day 4: Protein Quality Analysis using UV-Visible Scan (pg.# 3 of 4)

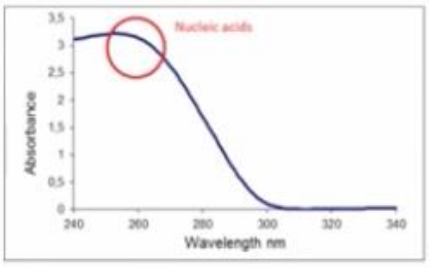
Procedure: Sample Measurements

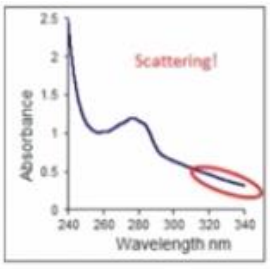
1. Prepare **100 µL** of protein sample in the concentration range of 1-2 mg/mL by diluting with the final buffer used for protein purification (gel filtration buffer).
2. Mix well by pipetting and **spin down** for **30 seconds** at **13,000 rpm**.
3. Clean the sample pedestal of the **Thermo Fisher NanoDrop™ 2000**, using **wet soft tissue wipe** followed by dry soft tissue wipe and **keep the arm in down position**.
4. Open the **NanoDrop™ program** on computer and wait for calibration to complete.
5. Start the **Protein A280 program** by clicking the option on computer screen. (**Preset wavelength range is 220 – 400 nm**).
6. Clean the pedestal and arm by wiping with clean soft tissue wipe.
7. **Load 5 µL** of **sample** buffer on pedestal, lower the arm and **click Blank** for baseline measurement.
8. Clean the sample pedestal and **load 5 µL of protein sample**, click **measure** to perform the wavelength scan.
9. **Record** the following values from the measurement and save the scan profile for each sample in a pdf format. (Refer to example scans below).

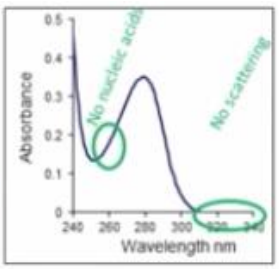
Desired value of A260/A280 < 0.6, for nucleotide free protein sample

Desired value of 100*A340/(A280-A340) < 2, for aggregate free protein sample.

Sample ID	A260	A280	A340	A260/A280	100*A340/ (A280-A340)







Date: _____ Day: _____

Day 4: SDS-PAGE Analysis of Protein (pg.# 4 of 4)

See Protocols 3A and 3B for making 17 % SDS-PAGE gels.

See Protocol 3C for rung the protein samples on SDS-PAGE gel.

See Protocol 3D for Silver staining the SDS-PAGE gel for protein size and purity analysis.

Protocol #2 Colony Screening for Protein Expression	
Materials	Bacterial transformation plate with colonies (from Protocol #1). LB-Kan+ media. 15 mL round bottom BD Falcon tubes. 1 M IPTG
Equipment	Spirit lamp, incubator-shaker.
Preparation	Thaw the LB-Kan+ media. Label 15 mL falcon tubes as #1, 2, 3 for colonies to be screened for expression.
Procedure	
1	Aliquot 5 mL of LB-Kan+ media in 3 Falcon tubes.
2	Pick a single colony, using tip of 10 µL pipette and transfer to a LB-Kan+ media tube #1. Repeat Step 2 to pick 2 more colonies and transfer to LB-Kan+ media in tubes #2 and #3.
3	Incubate the culture by shaking at 220 rpm and 37ºC until OD600 = 0.5
4	Aliquot 500 µL of culture and add 500µL of DI water. Mix well and measure OD600. Use dilution factor of 2 to get OD600 of original sample. Pre-Induction Sample: follow Steps 8 and 9
5	Aliquot another 500 µL of culture in an autoclaved eppendorf tube, and make glycerol stock by following Steps 10-12, below.
6	Induce remaining 4 mL of culture by adding 0.8 µL of 1M IPTG stock solution. Induce at 30 ºC with shaking, for 6 hours.
7	Aliquot 500 µL of culture after induction. Dilute with 500 µL DI water and measure OD600. Use dilution factor of 2 to get OD600 of original sample. Post-Induction Sample: follow Steps 8 and 9.
8	Spin down the cells at 13,000 rpm for 2 minutes and discard the supernatant. Resuspend the pellet in 50 µL of 8 M urea and add 50 µL of 2x SDS loading dye mix well. Boil the sample for 5 minutes at 95ºC. Run sample volume equivalent to OD600 of 0.15 units for original culture on 17% SDS-PAGE gels (See protocol # 3 below)
9	Spin down cells at 5000 rpm for 1 minute. Discard the supernatant and resuspend the cells in 400 µL of fresh LB-Kan+ media. Add 100 µL of autoclaved, 80% glycerol, mix well by pipetting up and down.
10	Aliquot 100 µL glycerol stock in 5 tubes. Label each tube with details of plasmid, cells, antibiotic resistance, date, name and colony number. Place the tubes in freezer box and save at -80ºC.
After analyzing expression test results on SDS-PAGE, discard the glycerol stocks with lower protein expression.	

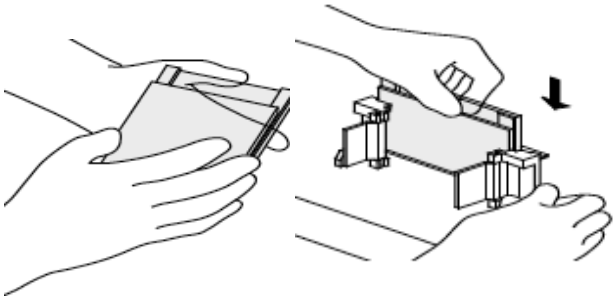
Additional protocols and information

Protocol #1 Bacterial Transformation for Protein Expression	
Materials and Equipment: BL21 (DE3) competent E. coli cells (from Stratagene/Agilent Cat# 200131). Plasmid DNA stock solution at 10 ng/µL concentration. 500 µL of 2x-YT broth (aliquots frozen at -20ºC), LB-Agar-Kan+ plates. Glass spreader, spirit lamp, 42ºC water bath, ice bucket, shaker, and 37ºC incubator. **Preparation:** Fill the bucket with ice and take the competent cells out from -80ºC freezer and place immediately on ice. Let the cells thaw completely (15 minutes). Check by inverting gently. Do not shake or vortex. Get the plasmid DNA tube and keep that on ice, bring the ice bucket to your bench. Turn on the water bath to maintain the temperature at 42 ºC. Incubate the 2x-YT broth in warming water bath. Label an autoclaved 1.5 mL eppendorf tube with the name of plasmid construct and chill on ice for 10 minutes.	
Procedure	
1	Aliquot 50 µL of BL21 (DE3) competent E.coli cells to a pre-chilled eppendorf tube.
2	Invert the tube 2 times and then spin down the plasmid solution at 13,000 rpm for 30 seconds.
3	Pipette 2 µL of plasmid (10 ng/µL stock concentration) and add to the 50µL competent cells. Mix by pipetting up and down gently 2 times. Close the lid and incubate the cells on ice for 30 minutes.
4	Transfer the eppendorf tube to a floating rack quickly. Place the floating rack in 42 ºC water bath for heat shock, for **45 Seconds**.
5	Transfer the tube back to ice bucket and incubate for 2 minutes.
6	Add 500 µL of pre-warmed 2x-YT broth. Close the lid, mix by gently inverting twice. Incubate the cells in 37ºC incubator with shaking at 220 rpm, for 1 hour
7	Sterilize the bench top by spraying 70% ethanol and wipe clean with paper towel. Get the LB-Agar-Kan+ plate (kanamycin: 50 µg/mL) from 4ºC fridge. Label the bottom of plate with name of plasmid, competent cells used, data, and name.
8	Ignite the spirit lamp and sterilize the glass spreaders by dipping in 70% alcohol and holding in flame for 2-3 seconds. Set the glass road aside to cool down (keep near the flame).
9	Spin down the cells at 5000 rpm for 2 minutes and discard 450 µL of supernatant. Resuspend the cells in remaining 100 µL media by pipetting up and down gently.
10	Transfer the cells to the center of agar surface using a pipette. Get the pre-sterilized and cooled glass spreader and touch to the agar surface near edge of the plate to confirm that it is cool. Spread the cells using spreader evenly across the entire surface of agar. Close the lid and keep the plate in the same position for 5-10 minutes

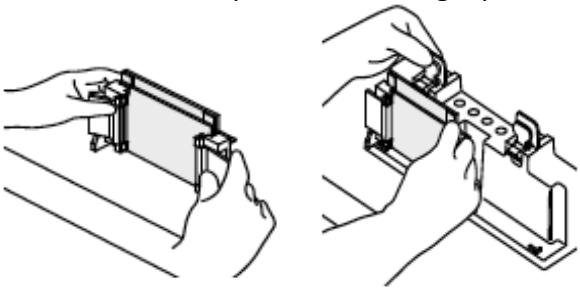
11	Sanitize the glass spreader by dipping in 70% alcohol contained in a beaker and then holding it in flame for 3 seconds.
12	Transfer the plate to the 37ºC incubator and place it upside down. Incubate overnight (15 hours).
13	Count the number of colonies. Perform colony screening for protein expression.

Protocol #3A Gel Plate Assembly to Pour the Gels

1. Clean the glass plates by spraying 70% alcohol and wipe using soft tissue wipes to remove any oily residues.
2. Place the BioRad gel casting frame upright on even surface, and clamps in open position.
3. Hold the spacer plate (0.75 mm thick spacer) in a way that the spacers are facing you and the BioRad name is at the top.
4. Place the Short plate on the spacer plate and align the bottom edges. The top part of spacer plate remains uncovered by short glass plate.
5. Insert the pair of aligned glass plates inside the small space of casting frame. Push the pair of plates all the way down so that the bottom edges of plates touch the leveled surface.



6. While holding plates together in position, close the side clamps of holder to secure the plates tightly in position.
7. Hold the clamp of casting stand open, and place the casting frame into casting stand, such that the bottom of gel plates rest firmly on the gray foam strip.
8. Release the clamp to secure the gel plates with clap pressure on the stand.

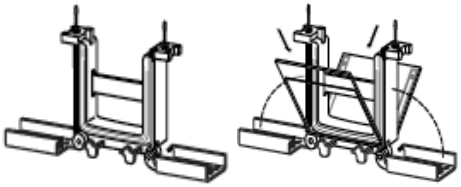


9. Assemble 12 pairs of gel plates in similar manner.
10. Gel assembly is read to pour the gels.

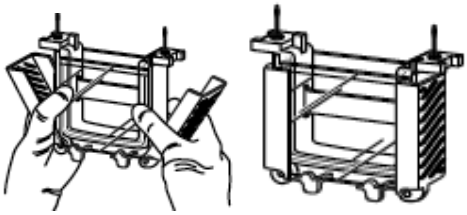
Protocol #3B Making SDS-PAGE Gels					
For 12 Gels	**Stacking**	**Resolving (running) Gel**			
Gel Percentage	5%	7.5%	12%	17%	21%
Autoclaved Water (mL)	12.24	25.92	17.82	8.7	1.62
30% Polyacrylamide (mL)	3.06	13.5	21.6	30.6	37.8
1.5 M Tris, pH 8.8 (mL)	----------	13.5	13.5	13.5	13.5
0.5 M Tris, pH 6.8 (mL)	2.25	---------	----------	----------	----------
1. Mix all the contents above in small beaker. 2. Filter using a Whatman™ filter paper disc (2 cm diameter) placed inside a Buchner funnel attached to a filter flask and vacuum pump. 3. Stir using magnetic stir bar while degassing for 5 minutes. 4. Stop degassing, and add following set of reagents slowly without introducing bubbles. Stir for additional 20-30 seconds.					
10% SDS (mL)	0.18	0.54	0.54	0.54	0.54
10% Ammonium persulfate* (µL)	96	540	540	336	540
Make fresh every time by weighing 0.1 g APS and dissolve in 1 mL DI water in 1.5 mL eppendorf tube. Dissolve by vortexing for 20-30 seconds.					
TEMED (µL)	18	54	54	54	54
5. Take the flask out from stirring plate and pour the resolving gel between assembled gel plates up to the mark (see plate assembly Protocol #3A for details). 6. Add n-butane as top layer to cover the poyacrylamide solution poured between plates. 7. Let polymerize for 2 hours. 8. Decant the n-butane in sink and rinse the plates using DI water. 9. Soak the residual water up by inserting the pieces of paper between plates. 10. Mix the contents for Stacking gel by following steps 1-4 above. 11. Pour the stacking gel on top of resolving gel between plates. 12. Insert the clean, well-forming comb between the glass plates. Let polymerize for 1 hour. 13. Remove the gel plates (with polymerized gels and combs inserted in) from the apparatus. Wrap individual gel set in paper towel to cover it from all the sides. Wet the paper towel by spraying DI water. 14. Keep individually wrapped gels in closely tight container. Label the container with the gel type, percent of running gel and date. Keep the box at 4 ºC until use.					

Protocol #3C Running SDS-PAGE Gels

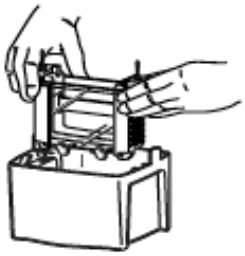
1. Open the electrode assembly closure clamps on either side and places it on even surface.
2. For running 2 gels, place the gel plate on either side of the electrode assembly, such that the short plates on gels are facing inwards.



3. Press the edges of gel plates against the green gasket of the electrode assembly.
4. Align the notches at top of the gasket with the top edge of short plate on both sides. Hold both plates in place with pressure and close the side clamps to secure the plats in position.



5. Insert the gel assembly in buffer tank by matching the electrode color codes and fill the gap between gels and buffer tank with 1X gel electrophoresis buffer.



6. Leave assembly for 10-15 minutes to check for buffer leakage between plates.
7. Take the comb out from both gel sandwiches and remove air bubbles by pipetting out.
8. Load 5 µL of sample in each well along with 5 µL of protein size marker on left most lane.
9. Cover the apparatus by connecting the appropriate electrode points from lid and run the gel in 2- stages

Step-1: 60 V for 30 minutes

Step-2: 140 V for 1 hour, 40 minutes.

Protocol #3D Silver Staining of Gels	
Read the Full Protocol Before Starting!	
Materials	Fixer Solution: 40% Ethanol, 10% Acetic Acid, 50% H_2O. Sensitizer Solution: 0.02% Sodium thiosulfate. 0.1% Silver nitrate solution. 35% Formaldehyde solution. Developing Solution: 3% Sodium carbonate. Terminator Solution: 5% Acetic acid.
Precautions	Use high quality chemicals and ultra pure water (18.2 MΩ·cm). Agitate the gels and perform work in a fume hood. Wear clean protective gloves at all stages. Use clean staining trays with lids, to avoid keratin contamination of the gels.
Procedure	
1	Incubate the gel in Fixer solution for 1 hour, with rocking in fume hood.
2	Wash the gel in H_2O for 30 minutes with water changes at every 10 minutes interval (10min X3). Overnight Washing with several water changes will remove all acetic acid, reduce background staining and increase sensitivity.
3	Sensitize the gel in 0.02% sodium thiosulfate solution for **1 min.**
4	Wash in H_2O for 20sec X 3.
5	Incubate gel for 20 min in 4ºC cold 0.1% silver nitrate solution containing 0.02% formaldehyde (add 40 µL of 35% formaldehyde to 70mL silver nitrate just before use).
6	Wash the gel in H_2O for 20sec X 3.
7	Place the gel in new staining tray and wash the gel in H_2O for 1min.
8	Develop the gel in 3% sodium carbonate, 0.05% formaldehyde. (Add 100 µL of 35% formaldehyde to 70 mL sodium carbonate just before use).
9	Terminate QUICKLY by washing with H_2O when staining is sufficient followed by incubation in 5% acetic acid for 5 min.
10	Leave the gel at 4ºC in 1% acetic acid for storage.

Protocol #4 M9 Minimal Medium

M9-Salt Solution

Salt	Weigh for 1L	Weigh for 8L	Formula Weight (g/mol)	Final Concentration (mM)
Na_2HPO_4	6.0 g	48.0 g	141.96	42.26
KH_2PO_4	3.0 g	24.0 g	136.086	22.04
NH_4Cl	1.0 g	8.0 g	53.491	18.69
NaCl	0.5 g	4.0 g	58.44	8.56
Add water to dissolve	900 mL	1800 mL	18	-
Note:	Make final volume to 1 L.	For 8 L, make final volume to 4 L and divide 1L in 4 flasks, add 1L water to each flask, mix well. Pouring 1L from each flask, back into 4 L plastic bucket, mix well. Repeat mixing in flasks and bucket to make homogenous, 8 L solution. Measure 1 L and divide in 8 flasks.		

Weigh all the solids and transfer to a clean beaker. Add 900 mL of D.I. water and dissolve solids by stirring on magnetic stir-plate. Make the final volume to 1 L in a graduated cylinder (calibrated) and transfer salt solution to a clean, 2 L media flask. Cover the flask mouth with aluminum foil, place autoclave tape. Place the flask in plastic tub containing D.I. water at the bottom. Sterilize by autoclaving for 25 minutes at 121ºC and 15 psi, on liquid cycle.

After sterilization, keep media on bench to cool down to room temperature. (Do not add following stock solutions if autoclaved solution is still hot).

Mix by swirling after addition of each stock solution.

Transfer the 5mL of starter culture to each 1 L M9-media flask, swirl to mix and cover the mouth of flask with the aluminum foil. Transfer the flask to shaker, set shaker temperature to 37ºC, and rotation

Before inoculation add following filter sterilized stock solutions

Material	Stock Concentration	For each L of M9-salt solution, add following	Formula Weight (g/mol)	Final Concentration
D-Glucose	20 %	10 mL	180.1559	0.2 %
$MgSo_4$	1 M	2 mL	120.366	2 mM
$CaCl_2$	1 M	0.1 mL (100µL)	110.98	0.1 mM
Kanamycin	50 mg/mL	1 mL	-	50 µg/mL

Protocol #5 Autoclave the Media	
1	Check if the autoclave is ready to use. (If generator needs to be flushed follow steps #6-8 below).
2	For sterilization of 8 L, M9 media, place 3 flasks in 2 plastic tubs and 2 flasks in third plastic tub. Fill each plastic tub with D.I. water up to an inch from the bottom. Place 3 tubs on the autoclave rack.
3	Close the door and check that flasks or tubs do not interfere with closing and opening of the autoclave door.
4	Select main menu then select Liquid cycle to sterilize the media for 25 minutes at 121 ºC and 15 psi pressure. Stay and observe that autoclave has started normally and there are no errors.
5	Upon completion of cycle, check the pressure before opening the door of autoclave. (Do not open immediately after cycle completion, as hot steam can rush outwards). Open the door, and remove the media flasks using heat protective gloves. Keep all the flasks on clean cart and let cool to room temperature before proceeding to next step for expression.
6	**Flushing the generator** – Close the door of the autoclave chamber by pressing the pedal. Open the front doorframe by disengaging the magnetic pull on the left center of the doorframe.
7	Turn the orange knob (located at bottom left corner) to open the generator valve. On the display, press start timer to start flushing the generator. It will take 5 minutes to flush (keep the doorframe open during flush cycle).
8	Turn the orange knob to close the generator valve, close the doorframe and press continue on the display panel to go to main menu. Autoclave is ready to use now.

Protocol #6 Ni-NTA Column Preparation and Cleaning	
Materials	Ni-NTA Agarose, pre-charged resin in 30% ethanol. (Catalog # 30230 for 100 ml) 100 mM $NiSO_4$, pH8.0 100 mM EDTA, pH 8.0 6 M Guanidium hydrochloride, in 200 mM acetic acid 500 mM NaOH 30% Ethanol
Equipment	Pierce™ Disposable Columns, (10 mL), matching frit discs and caps. (Catalog # 29924) PharMed Tubing (BioRad Catalog #7318207)
Prepare the Ni-NTA Affinity Column for Purification	
1	Place the frit disc inside the polypropylene column and push to the bottom using packing plunger device. Attach flexible PharMed tubing at the bottom of the column and clamp using small binder clip. Secure the column on a clamp attached to the ring stand. Pour D.I. water to wet the inside of column and the frit disc. And let flow with gravity by opening the binder clip.
2	Clamp the outlet tube and add 2 mL of D.I. water into the column using 1 mL micropipette. Make a 2 mL mark using a marker pen. Let the water flow through to empty the column.
3	Mix the Ni-NTA agarose resin by swirling the bottle gently. Use 1 mL pipette to add resin slurry to the column until the packed bed volume is up to 2 mL mark.
4	Wash the resin using 20 mL of D.I. water. Clamp the bottom tube and add 2 mL of D.I. water to keep the resin wet. Column is now ready for equilibration.
5	Secure the cap on top of the column and store at 4 ºC until ready to use.
Cleaning and Recharge after Use	
1	Wash the column with 10 mL of D.I. water.
2	Add 5 mL of 500 mM NaOH and leave for 30 minutes.
3	Wash the column with 20 mL of D.I. water.
4	Wash the column with 5 mL of regeneration buffer (6 M GuHCl in 200 mM acetic acid).
5	Wash the column with 20 mL of D.I. water.
6	Wash the column with 10 mL of 100 mM EDTA, pH 8.0 and collect the flow through in separate Ni-Waste container. (Ni-waste is toxic and need to be stored in separate, labeled container).
7	Wash the column with 20 mL of D.I. water.
8	Recharge the column by adding 5 mL of 100 mM $NiSO_4$ solution. Leave the $NiSO_4$ solution in the column until next use ($NiSO_4$ prevents bacterial growth).
Using after Recharge	
1	Drain the $NiSO_4$ solution from column in Ni-Waste container. Wash with 5mL of D.I. water and collect the flow through in Ni-waste container.
2	Wash the column with 10 mL of D.I. water.

3	Equilibrate with 5 mL of a lysis buffer. Note: Store refrigerated at 4 ºC to inhibit growth of microorganisms for long-term storage. Ni-NTA matrices can be stored in 30% ethanol to inhibit microbial growth

Protocol #7A Centrifuge Usage with F10S Rotor for Pelleting Cells	
Spin down cells using FS-10 rotor, for 8 minutes at 5000 rpm at 4 ºC	
1	Place FS-10 rotor on to the spindle carefully, without tilting. Turn the main switch on.
2	Check the correct sizes of all the centrifuge bottles to be used in this rotor. (FS-10 rotor can fit 6, 500 mL bottles). Examine bottle for cracks or broken lids. **Do not use cracked bottles or lids.**
3	Pour the cell culture up to 3/4th height of each bottle (~350 mL) and balance bottles with their matching lids, within ±0.05 g difference. (Use D.I. water to adjust small weight difference).
4	Dry the outside of the bottles with paper towel and place them in the FS-10 rotor.
5	Place the rotor lid and tighten it for securing the rotor in place. Test security of the rotor by lifting from bottom to make no movement is detected.
6	Close centrifuge lid, Select the rotor FS-10 (rotor code = 47), Enter speed = 5000 rpm, Time = 8 minutes, Temperature = 4 ºC.
7	Press the 'Start' button. Stay and obverse until the desired speed is reached. Make sure no noise or vibration is detected. If abnormal noise or vibration is detected, press the 'Stop' button immediately and recheck everything.
8	An alarm will ring indicating that centrifugation cycle is complete. Let the rotor make complete stop before opening the centrifuge lid (rotor motion and speed shows in display panel). Loosen rotor lid and keep the lid on even surface on a bench. Take centrifuge bottles out, and keep on the cart. Discard the supernatant in the sink.
9	Repeat step #3-8, until all the cells are spun down. Keep the cell pellet on ice before proceeding to the next step (cell resuspension and lysis).
10	Take the rotor out. Clean any spills or condensation by wiping it dry. Place the rotor and lid properly on an even bench surface.
11	Switch the power off and leave centrifuge door open to allow drying.

Protocol #7B Centrifuge Usage with SS34 Rotor for Spinning Lysate	
Spin down lysate using SS34 Rotor, for 30 minutes, 15,000 rpm at 4 ºC	
1	Place SS-34 rotor on to the spindle carefully, without tilting. Turn the main switch on. Close the door and let the temperature reach to 4 ºC before spinning the lysate.
2	SS-34 rotor can fit eight 50 mL, high-speed centrifuge tubes to spin down the cell lysate.
3	Transfer the lysate from a beaker to centrifuge tubes up to 3/4th height (~30mL). Use small Styrofoam holder to keep tube upright for balancing. Balance all tubes with their lids to be within ±0.05 g difference. Adjust the weights using the lysis buffer.
4	Dry the outside of the tubes with paper towel and place them in the SS-34 rotor.
5	Place the rotor lid and tighten it for securing the rotor in place. Test security of the rotor by lifting from bottom to make no movement is detected.
6	Close centrifuge lid, Select the rotor SS-34 (rotor code = 5), Enter speed = 15,000 rpm, Time = 45 minutes, Temperature = 4 ºC.
7	Press the ‘Start’ button. Stay and observe until the desired speed is reached. Make sure no noise or vibration is detected. If abnormal noise or vibration is detected, press the ‘Stop’ button and recheck everything.
8	An alarm will ring indicating that centrifugation cycle is complete. Let the rotor make complete stop before opening the centrifuge lid (rotor motion and speed shows in display panel).
9	Loosen rotor lid and keep the lid on even surface on a bench. Take centrifuge tubes out, and keep on the rack and take to your bench to proceed for next step (for protein purification by Ni-affinity chromatography).
10	Take the rotor out. Clean any spills or condensation by wiping it dry. Place the rotor and lid properly on an even bench surface.
11	Switch the power off and leave centrifuge door open to allow drying.

Protocol #8 Ni-NTA Affinity Chromatography Buffers

Lysis Buffer	Concentration	Formula Weight (g/mol)	Amount per Liter (g)
NaH_2PO_4	50 mM	137.99	6.90
NaCl	500 mM	58.44	29.22
Imidazole	-	68.08	-
Procedure: 1. Adjust pH to 8.0 using NaOH (solid pellets). Make final volume to 1L using a graduated cylinder. 2. Filter through 0.2 µm membrane filter using vacuum filtration apparatus. 3. Transfer to a dry, autoclaved glass bottle. Label the bottle and store at 4 ºC fridge.			

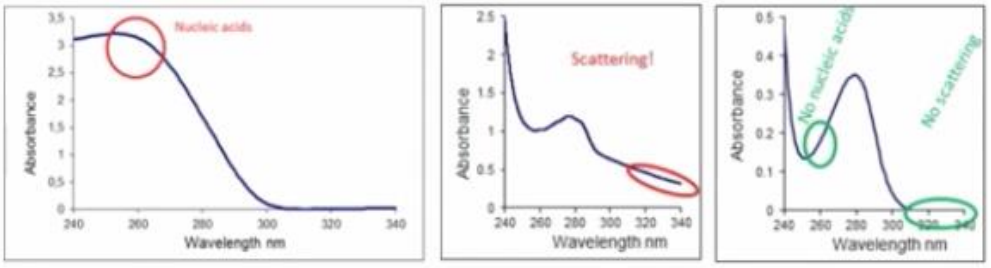
Wash Buffer	Concentration	Formula Weight (g/mol)	Amount per Liter (g)
NaH_2PO_4	50 mM	137.99	6.90
NaCl	500 mM	58.44	29.22
Imidazole	80 mM	68.08	5.44
Procedure: 1 Adjust pH to 8.0 using NaOH (solid pellets). Make final volume to 1L using a graduated cylinder. 2 Filter through 0.2 µm membrane filter using vacuum filtration apparatus. 3 Transfer to a dry, autoclaved glass bottle. Label the bottle and store at 4 ºC fridge.			

Elution Buffer	Concentration	Formula Weight (g/mol)	Amount per Liter (g)
NaH_2PO_4	50 mM	137.99	6.90
NaCl	500 mM	58.44	29.22
Imidazole	250 mM	68.08	17.02
Procedure: 1 Adjust pH to 8.0 using NaOH (solid pellets). Make final volume to 1 L using a graduated cylinder. 2 Filter through 0.2 µm membrane filter using vacuum filtration apparatus. 3 Transfer to a dry, autoclaved glass bottle. Cover the bottle with aluminum foil, place label and store at 4 ºC fridge.			

Protocol #9 Cell Lysis using Avestine C3 Emulsiflex Homogenizer	
1	Switch the homogenizer on, turn on nitrogen (80 psi on pressure manifold)
2	Unscrew the cap and check to make sure ethanol has not evaporated.
3	Turn red Stop knob clockwise and then push green button to Start.
4	Pump residual ethanol out of the tube and rinse with 50 mL DI water.
5	Add ~30 mL of 2-5% Contrad 70 soap solution and circulate it through homogenizer. When the outlet tube is free of bubbles, apply pressure by turning the air regulator (gray) knob, clockwise. Air pressure reads ~50-60 psi and gauge pressure reads between15, 000 and 20, 000 psi.
6	Circulate soap solution from outlet tubing to the sample cylinder for one minute at applied pressure. Lower the pressure and let soap solution flow out to waste container.
7	Repeat Steps 5 and 6 using 400 mL of warm water (200 mL X 2) followed by 200 mL DI water.
11	Remove the sample cylinder and keep on ice for 10 minutes.
12	Fill the plastic tub with icy water. Start circulating chilled water through 'Heat Exchanger' by turning on the submersible water pump, placed in icy-water.
13	Rinse sample cylinder with DI water and install back to the top.
14	Load your samples into the sample cylinder and direct the outlet tubing back to the cylinder.
15	Turn on the homogenizer by turning red stop knob and then pressing the green start button. Let the samples run through the tubing back to the cylinder to make sure, the flow is continuous and there are no bubbles in tubing.
16	Turn the air pressure on by turning the gray knob clockwise. Air pressure at 40-60 psi, gauge pressure ~15,000 psi and 20,000 psi.
17	Circulate the sample through homogenizer for 2 minutes (for 30 mL sample) with applied pressure. (Circulate sample longer for larger volumes; 2 min for each 30 mL of sample).
18	Collect the lysate by transferring the outlet tube to a clean beaker chilled on ice.
19	Lower the pressure to zero when sample is not visible inside the sample cylinder and collect residual sample in beaker.
20	Press red stop knob. Turn off the water-circulating pump. Follow steps 5-7 to clean the homogenizer and piston.
21	Pour ~50 mL of 50% ethanol and let it flow through to fill the outlet tubing. Press red stop knob. Turn of the nitrogen. Screw the lid back to sample cylinder and turn main switch off.

Protocol #10
Protein Quality Analysis using UV-Visible Scan on NanoDrop™

1 Prepare 100 µL of protein sample in the concentration range of 1-2 mg/mL by diluting with the final buffer used for protein purification (gel filtration buffer).
2 Mix well by pipetting and spin down for 30 seconds at 13,000 rpm.
3 Clean the sample pedestal of the Thermo Fisher NanoDropTM 2000, using wet soft tissue wipe followed by dry soft tissue wipe and keep the arm in down position.
4 Open the NanoDrop™ program on computer and wait for calibration to complete.
5 Start the Protein A280 program by clicking the option on computer screen. (Preset wavelength range is 220 – 400 nm).
6 Lift the arm of pedestal and load 5 µL of sample buffer. Place the arm down and click Blank for baseline measurement.
7 Clean the pedestal and arm (top and bottom tip contacting the sample) by wiping with clean soft tissue wipe.
8 Load 5 µL of protein sample, lower the arm and measure the absorbance by scanning the buffer.
9 Note: Use appropriate buffer as blank for different set of protein samples.
10 Perform triplicate measurements for each sample.
11 Clean the sample pedestal and repeat the measurement for protein sample (as step 10 above).
12 Record the following values from the measurement and save the scan profile for each sample in pdf format. (Refer to example scans below).



Appendix B

Real-time *In Vitro* Fluorescence Anisotropy of the Cyanobacterial Circadian Clock

Abstract

Uniquely, the circadian clock of cyanobacteria can be reconstructed outside the complex milieu of live cells, greatly simplifying the investigation of a functioning biological chronometer. The core oscillator component is composed of only three proteins, KaiA, KaiB, and KaiC, and together with ATP they undergo waves of assembly and disassembly that drive phosphorylation rhythms in KaiC. Typically, the time points of these reactions are analyzed ex post facto by denaturing polyacrylamide gel electrophoresis, because this technique resolves the different states of phosphorylation of KaiC. Here, we describe a more sensitive method that allows real-time monitoring of the clock reaction. By labeling one of the clock proteins with a fluorophore, in this case KaiB, the *in vitro* clock reaction can be monitored by fluorescence anisotropy on the minutes time scale for weeks.

Keywords: Fluorescence; circadian clock; cyanobacteria; protein; phosphorylation

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Reprint

Conceptualization, J.H. and A.L.; Methodology, J.H. and A.L.; Formal Analysis, J.H.; Writing--Original Draft Preparation, J.H, and A.L.; Writing--Review & Editing, J.H, A.C., and A.L.; Supervision, A.C., Y.G.C, and A.L.; Funding Acquisition, A.L

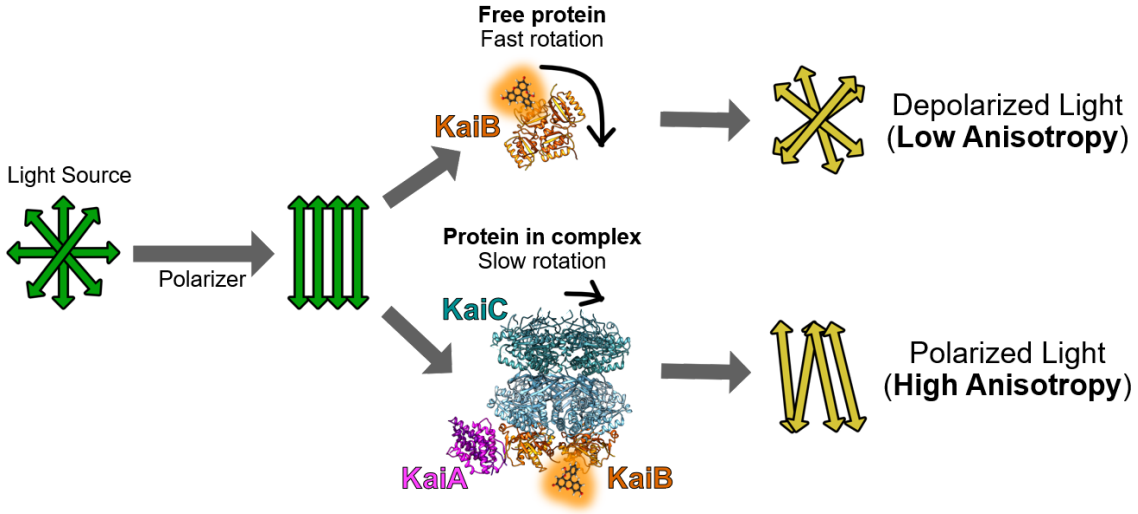
1. Introduction

Organisms from all domains of life display circadian (~24 h) rhythms in their metabolism, physiology, and behavior that arose as an adaptation to daily cycles of ambient light and temperature.[40] These endogenous rhythms are generated by intracellular circadian clocks. Despite extensive investigations in fungi, plants, insects, and vertebrates, the mechanistic nature of circadian clock protein-protein interactions remains mysterious. Among model systems, the cyanobacterial clock offers a unique opportunity in this regard. It can be reconstituted *in vitro* by simply mixing its three protein components—KaiA, KaiB, and KaiC—with ATP, resulting in a macroscopic ~24 h rhythm of KaiC phosphorylation.[31]

Just as a watchmaker's apprentice learns the mechanism of a watch by studying its gears as they move, it is informative to observe components of the cyanobacterial clock as they move. Typically, time points of *in vitro* cyanobacterial clock reactions are analyzed ex post facto using denaturing polyacrylamide gel electrophoresis (SDS PAGE) to resolve different states of KaiC phosphorylation. It was used to resolve the ordered temporal pattern of KaiC phosphorylation: S/T→S/pT→pS/pT→pS/T→S/T→…, where S and T represent residues S431 and T432, the two phosphorylation sites of KaiC, and pS and pT denote their phosphorylated states.[68,69] This method has allowed for numerous insights into the cyanobacterial clock, such as KaiA stimulates KaiC autophosphorylation during the day, and KaiB inhibits KaiA in order to promote KaiC autodephosphorylation at night.[39,46,50,67,78,124] However, it has some inherent disadvantages. For example, reactions cannot be monitored in real time. Removing aliquots from reactions every few hours followed by SDS PAGE limits how many experiments can be run in parallel and the duration of each experiment. Electrophoresis and densitometry of stained gels to determine KaiC phosphorylation levels is a manual process. In addition, this SDS PAGE approach has low temporal resolution (hour time scale), ≥10% uncertainty in KaiC phosphorylation levels per time point, does not directly inform on protein-protein interactions, and perturbs the sample (by taking time points). However, the recent development of an automated sampling device could reduce some of these drawbacks.[159]

Here, we demonstrate that *in vitro* fluorescence spectroscopy can directly monitor circadian rhythms of protein-protein interactions in the cyanobacterial clock in real time by utilizing clock proteins labeled with fluorescent dyes. This methodology uses a fluorescently-labeled construct of KaiB,[121–123] and the plasmid construct for expressing KaiB-FLAG-K25C (described in the Sections 2.1.1 and 3.1) is available to the scientific community. It is worth mentioning here that real-time measurement of luciferase-based bioluminescence *in vivo* is a

powerful method for investigating circadian gene expression rhythms in cyanobacteria.[24] The availability of crystal structures of the free clock components and their complexes make it feasible to select fluorophore labeling sites that do not perturb the system.[56] As will be demonstrated below, tracking fluorescence anisotropy of 6-iodoacetamidofluroescein (6IAF) labeled KaiB allows direct observations of real-time population shifts between free KaiB (daytime) and bound KaiB (nighttime) (Appendix B Fig.1). This fluorescence method is straightforward, does not perturb the sample during measurements, and offers a high time resolution (minutes) of the clock as it ticks.



Appendix B Fig.1. Cartoon of how labeled-KaiB protein free (top) and in ternary KaiA-KaiB-KaiC complexes (bottom) have significantly different fluorescence anisotropies. Rotational correlation times of the fluorophore can be extrapolated following a calibration of fluorescence anisotropy with neat fluorescein in the presence of glycerol, which has defined values.[111]

The methodology utilizes standard molecular cloning, protein expression, and labeling procedures. A convenient approach to site-directed DNA mutagenesis is Quikchange PCR, allowing for substitutions, deletions, or small additions with a single-step polymerase chain reaction conducted on a plasmid vector.[120] Then, standard heat shock transformation of *E. coli*, expression, purification of the protein and fluorophore labeling sets the stage for facile real-time measurements of protein-protein interactions in oscillating clock reactions. Appendix B Fig.2 provides structural insight into the selected KaiB conjugation site for fluorophore labeling, highlighting how lysine 25 is oriented away from the intramolecular

tetramer and dimer interfaces, and intermolecular KaiC and KaiA binding sites, allowing for conserved function of KaiB.

Fluorescence anisotropy is widely used by biochemists. Basically, shining polarized light on an isotropic solution selectively excites fluorophores whose transition moments are parallel to the direction of polarization.[110] Excited-state lifetimes of many commercially available fluorophores are similar to the rotational correlation times of proteins (nanoseconds), making fluorescence anisotropy of labeled proteins sensitive to protein-protein interactions (Appendix B Fig.1).

2. Experimental Design

2.1. Materials

2.1.1. Quikchange Preparation of the KaiB-K25C Construct for Fluorescence Labeling

KaiB-K25C plasmid construct[123] (available upon request)

10 ng/µL pET28b+ plasmid template DNA of *kaiB* gene (*see* **Note 1**).

DpnI restriction enzyme (10 units/µL) (New England BioLabs, Cat. no.R0176L, Ipswich, MA).

Quikchange PCR primers (*see* **Note 2**).

10× PfuTurbo C*x* reaction buffer (1.0 units/50 µL PCR) (Agilent Technology, Cat. no.600257).

PfuTurbo DNA polymerase (1.0 units/50 µL PCR).

10 mM deoxynucleotide triphosphates (dNTPs) solution mix (New England BioLabs, Cat. no.N0447L, Ipswich, MA).

(TAE) buffer: 1.5% agarose gel in Tris base (Fisher Chemical, Cat. no.BP152-10, Waltham, MA, USA), acetic acid (Fisher Chemical, Cat. no.A38-k212, Waltham, MA), and EDTA (Fisher Scientific, Cat. no.BP121-500).

Competent BL21-DE3 *E.coli* cells.

Luria Broth (LB) agar plates with 50 µg/mL kanamycin (ThermoFisher Scientific, Cat. no11815032, Waltham, MA) (*see* **Note 3**).

500 µL of 2× YT bacterial culture media broth per sample.

2.1.2. PCR Screening and DNA Sequencing

LB kan^+ media broth.

1.5% agarose gel in Tris base, acetic acid, and EDTA (TAE) buffer.

2.1.3. Protein Expression

Freshly transformed cells harboring the expression construct.

LB media to grow cells.

50 mg/mL kanamycin antibiotic.

1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (Research Products International, Cat. no.I56000-200.0, Mt Prospect, IL).

Lysis buffer: (50mM NaH_2PO_4 (Sigma-Aldrich, Cat. no.S3139-500G, St. Louis, MO), 500mM NaCl (Fisher Chemical, Cat. no.S271-10, Waltham, MA), pH 8.0) (*see* **Note 4**).

For proteins to be fluorescently labeled, tris(2-carboxyethyl)phosphine (TCEP) (GoldBio, Cat. no.TCEP25, St Louis, MO) was used to prevent unwanted disulfide bond formation between proteins (*see* **Note 5**).

2.1.4. Fluorescent Labeling of Clock Protein

Fluorescent dye compatible with thiol (-SH) click chemistry attachment (6-iodoacetamidofluorescein) (ThermoFisher Scientific, Cat. no.I30452, Waltham, MA) (*see* **Note 6**).

Reduced KaiB-K25C mutant construct with cysteinyl residue available for fluorophore labeling (wild-type KaiB has no naturally occurring cysteinyl residues).

Labeling buffer (20 mM Tris, 150mM NaCl, pH 7.0) (*see* **Note 7**).

Desalting column buffer for protein storage (20 mM Tris, 150 mM NaCl, pH 8.0).

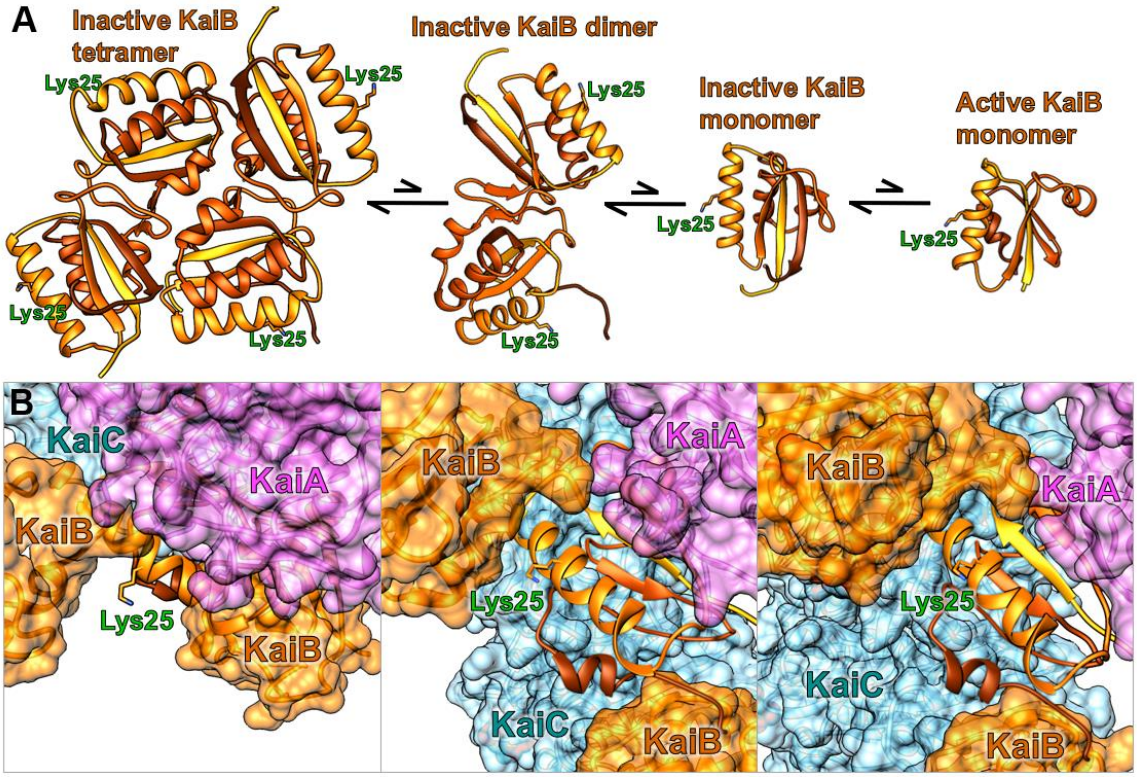
2.1.5. Fluorescence Anisotropy Binding Assay

Fluorescently labeled clock protein (*see* **Subheading 3.5**).

600 µL reaction buffer: (20 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$ (Fisher Scientific, Cat. no.BP214-500, Waltham, MA), 1 mM ATP (MP Biomedicals, Cat. no.194613O, Burlingame, CA), 0.5 mM EDTA, 50 µg/mL kanamycin, pH 8.0) for each reaction sample.

Unlabeled cyanobacterial clock proteins FLAG-KaiC, KaiA, KaiB-FLAG[84] (plasmids available upon request) (*see* **Note 8**).

Low protein-binding syringe filter (13 mm with 0.2 um membrane).



Appendix B Fig.2. Fluorescence labeling site lysyl 25 on KaiB in relation to (A) KaiB's four different free states and (B) ternary KaiABC complex shown in multiple orientations, with KaiA (purple), KaiB (orange), and KaiC (blue).[56]

2.2. Equipment

2.2.1. Quikchange Preparation of the KaiB-K25C Construct for Fluorescence Labeling

- Micropipettes (0.2–2 µL, 2–20 µL, and 20–200 µL) and pipette tips.
- DNA molecular size ladder (New England BioLabs, Cat. no.N3200L, Ipswich, MA).
- 0.2 mL individual PCR tubes with snap shut caps to hold PCR reactions.
- Thermocycler.

- Gel electrophoresis apparatus (BIO-RAD Wide Mini-Sub Cell GT and PowerPac universal power supply, Hercules, CA).
- 1.5 mL sterile centrifuge tubes.
- Ice bath (small bucket or tray).
- Water bath (set to 42 °C).
- Temperature controlled shaker (set at 37 °C).
- Glass rod for spreading BL21-DE3 transformed cells on LB agar plates.
- Alcohol lamp or Bunsen burner for sterile technique.
- Incubator (set at 37 °C) for cultured LB agar plates.

2.2.2. PCR Screening and DNA Sequencing

- Micropipettes (0.2–2 µL, 2–20 µL, and 20–200 µL) and pipette tips.
- 15 mL sterile Falcon tubes.
- DNA molecular size ladder.
- Thermo Scientific GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, Cat. no.K0503, Waltham, MA).
- Benchtop centrifuge.
- Gel electrophoresis apparatus (BIO-RAD Wide Mini-Sub Cell GT and PowerPac universal power supply, Hercules, CA).
- Thermo Scientific Sorvall Legend RT+ Centrifuge.
- Thermo Scientific NanoDrop UV-Vis 2000 Spectrophotometer.
- Temperature-controlled shaker at 37 °C and capable of 220 rpm.

2.2.3. Protein Expression

- Inoculating loop.
- Autoclave.
- UV-Vis spectrometer (HACH DR 6000EDU, Loveland, CO).
- Temperature controlled shaker (set at 37 °C).
- Centrifuge to harvest cells, and to separate protein supernatant from insoluble debris after cracking cells. We use a Thermo Scientific Sorvall RC 6 Plus Superspeed centrifuge. It has 4 l of capacity, speeds of up to RCF of 55,200 × g.

- Centrifuge rotors for 50 mL and 300 mL centrifuge bottles.
- Sterile volumetric pipet.
- Transfer bulb for volumetric pipet.
- Homogenizer for cell disruption (see Note 9).
- Nickel-nitrilotriacetic acid (Ni-NTA) gravity-flow chromatography columns for protein purification (10 mL polypropylene columns with QIAGEN Ni-NTA agarose beads) (Qiagen, Cat. no.30230).
- Amicon stirred cell and a 10 kDa cut-off ultrafiltration disc (Millipore Sigma, Cat. no.PLGC04310, St. Louis, MO).
- Benchtop centrifuge and 1.5 mL centrifuge tubes.
- Liquid chromatography 5 mL injection syringe, needle, and adaptor for injection into instrument.
- Fast protein liquid chromatography (FPLC) to collect protein eluates.
- GE Healthcare Life Sciences size-exclusion HiLoad 16/600 Superdex 75.
- Nanodrop to measure protein concentrations by Bradford Assay (see Note 10).

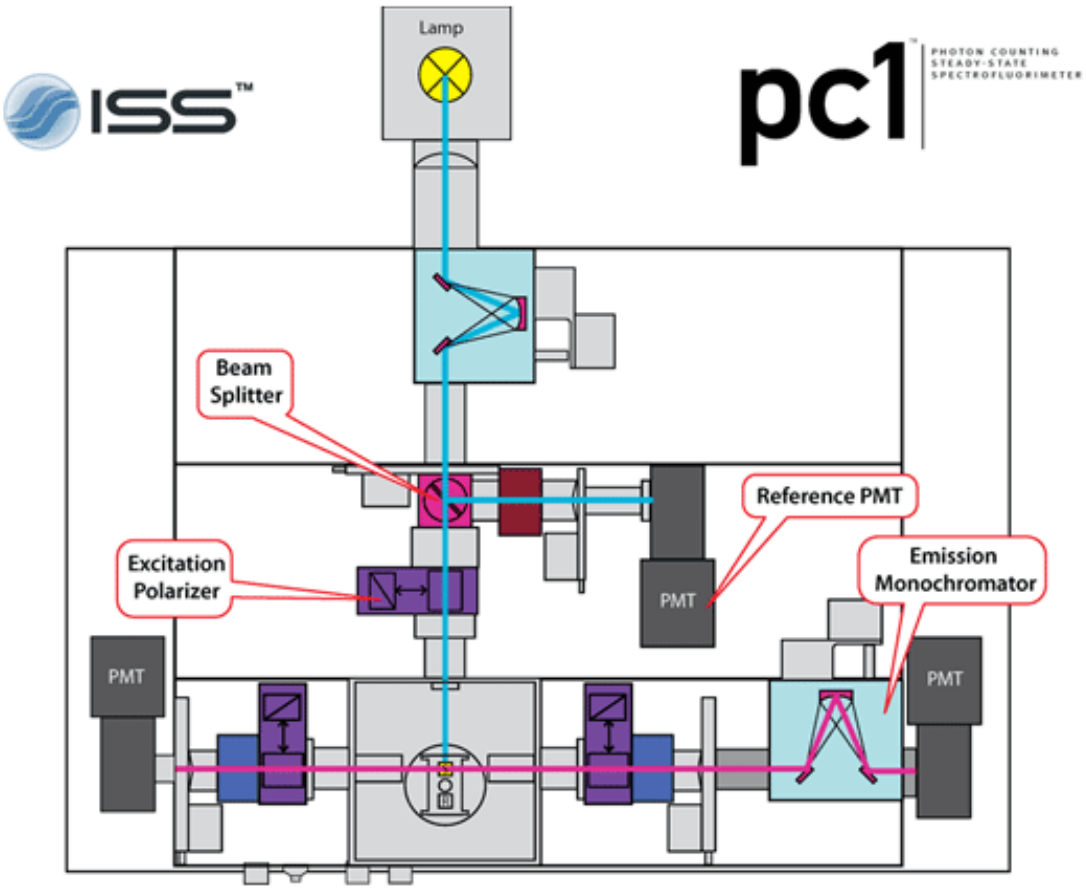
2.2.4. Fluorophore Labeling of Clock Protein

- Covered sterile Corning tube to hold protein sample during labeling reaction without light exposure (see Note 11).
- FPLC to separate labeled KaiB construct from free dyes.
- Stirred cell concentrator (see Note 12).
- Liquid chromatography 2 mL injection syringe, needle, and adaptor for injection into instrument.
- GE Healthcare Life Sciences HiPrep 26/10 desalting column.
- Nanodrop to measure protein concentrations by Bradford Assay.

2.2.5. Fluorescence Anisotropy Binding Assay

- Photon counting spectrofluorimeter (see Note 13).
- Three-cuvette sample compartment for fluorimeter.
- Circulating water bath at 30 °C and tubing compatible with fluorimeter for sample temperature control.

- 5 mm quartz cuvettes to hold reaction samples.
- Low protein-binding 0.2 µm membrane filter for each reaction sample (Pall Corporation, Cat. no.PN 4602).
- 1 mL syringe for sample filtration.
- Stock MgCl2 and ATP solutions (see Note 14).
- 1.5 mL sterile centrifuge tubes, three for each reaction sample (see steps 80–88).



Appendix B Fig.3. ISS PC1 instrument setup for polarization of incident light and detection of parallel and perpendicular polarized light after passing through the sample cuvette. This figure is from reference.[160] Here, once the sample is excited by polarized light, only the right instrument path is utilized, with the right photon multiplier tube collecting both parallel and perpendicular emissions in turn through software-controlled emission polarizer orientation adjustments (bottom right purple rectangle).

3. Procedure

3.1. DNA Sample Preparation using Quikchange PCR. (Time of Completion: 5–6 h)

1. Prepare Quikchange PCR solutions in PCR tubes (see Table B.1).

2. Setup thermo cycler to run program (see Appendix B Fig.4) (see Note 15).

3. Add 1 µL of DpnI enzyme to each Quikchange PCR product, and spin down in benchtop centrifuge at RCF of 18,000 × g for 1 min.

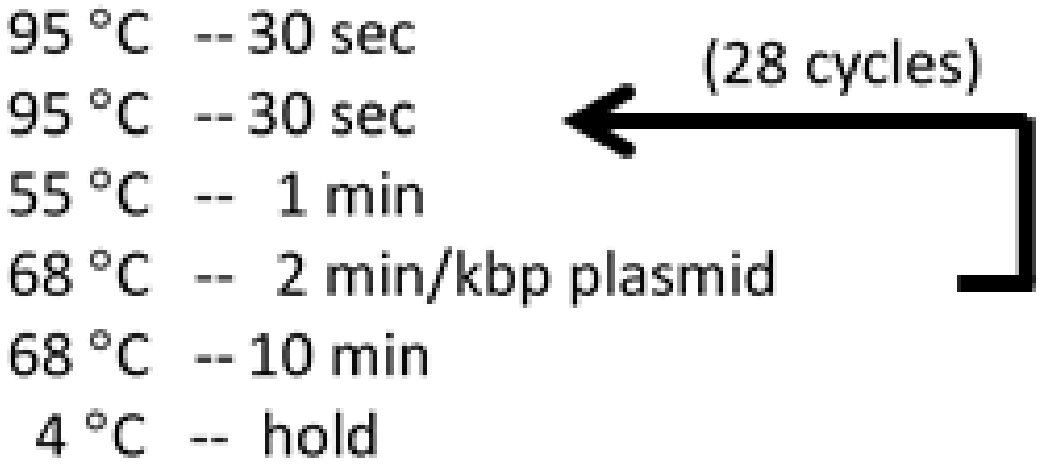
4. Incubate Quikchange PCR products with DpnI for 1 hour at 37 °C.

5. PCR purification kit, optional (QUIAGEN).

6. Measure Quikchange PCR product concentration with Nanodrop. Ensure the 260 nm/280 nm ratio is near 1.8 for pure DNA product (see Note 16).

Appendix B Table 1. Quikchange PCR reagents used in a generic reaction solution.

PCR Reaction	
5 µL (1×)	(10×) Pfu Turbo C*x* Reaction Buffer
5 µL (50 ng)	Template Plasmid (10 ng/µL)
1 µL (200 nM)	Primer 1 (10 µM)
1 µL (200 nM)	Primer 2 (10 µM)
10 µL (2.5 mM)	dNTP Mix (10 mM)
27 µL	ddH_2O
1 µL	Pfu Turbo DNA Polymerase (1 units⁄50 µL)
50 µL	Total Volume



Appendix B Fig.4. Temperatures and durations for Quikchange PCR thermo cycler program.

3.2. Transformation of Competent Cells with DpnI-treated Quikchange products. (Time of Completion: 1 day)

7. Chill centrifuge tubes on ice for 5–10 min.

8. Warm 500 µL of 2× YT broth for each transformation sample.

9. Add 50 µL of BL21-DE3 competent cells to each chilled centrifuge tube.

10. Add 2 µL of DpnI digested Quikchange PCR product, mix well and incubate on ice for 30 min (see Note 17).

11. Heat shock the cells at 42 °C in a water bath for 45 s.

12. Incubate them on ice for 2 min.

13. Add 500 µL of warmed 2× YT broth to each sample.

14. Incubate cells in temperature-controlled shaker at 37 °C and 220 rpm for 1 h.

15. Spin down cells at RCF of 2,660 × g for 1 min in benchtop centrifuge (at room temperature).

16. Discard 350 µL of supernatant and resuspend the transformed cell pellet with the remaining supernatant (150 µL).

17. Pipet 150 µL of resuspended cells per LB agar plate containing kanamycin (50 µg/mL).

18. Spread cells on agar surface using sterile glass spreader (see Note 18).

19. Incubate plates for 16–18 h at 37 °C (see Note 19).

3.3. PCR Screening and DNA Sequencing. (Time of Completion: 1 day)

20. Obtain transformation plate with colonies harboring mutant construct.

21. Prepare 15 mL Falcon tubes (three for each mutant) with 5 mL LB-kan+ broth media in each.

22. With an inoculating loop, and aseptic technique, pick a colony from the plate, and dip & swirl loop into a Falcon tube containing media for each. Do this step for three colonies.

23. Incubate samples for 4–6 h at 37 °C with shaking at 220 rpm (see Note 20).

24. Prepare PCR centrifuge tubes with 1 µL incubated transformation product, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), 10 µL dNTP (10 mM), 1 µL Pfu Turbo DNA Polymerase (1 unit/50 µL), 5 µL 10× Pfu Turbo Cx Reaction Buffer, and dilute to 50 µL with deionized water (see Table 1).

25. Run PCR thermo cycle to amplify transformed DNA sequence (see Appendix B Fig.4).

26. Prepare a 1.5% agarose gel.

27. Load 30 µL of PCR product into 1.5% agarose gel, along with 1 kbp DNA ladder, and run at 60 V for 20 min.

28. Analyze PCR product bands to confirm that the DNA sequence is both present at a reasonable concentration and the correct length.

29. Spin down remainder of cultures for 10 min at 4 °C and 4000 rpm using Thermo Scientific Sorvall Legend RT+ Centrifuge.

30. Discard supernatant and isolate DNA plasmid from pelleted cells using Thermo Scientific GeneJET Plasmid Miniprep Kit.

31. Measure DNA miniprep product with Nanodrop. Expect concentration of 100–300 ng/µL.

32. Prepare 500 ng of miniprep product in deionized water (10 µL total volume each).

33. Each sample requires two tubes, one with forward primer (0.5 µL at 10 µM) and the other with reverse primer (0.5 µL at 10 µM) added.

34. Send samples to DNA sequencing facility.

35. Analyze results to ensure desired mutations are present before moving forward with protein expression.

3.4. Protein Expression. (Time of Completion: 3 days)

3.4.1. LB media. (Time of Completion: 2 h)

36. Add 25 g LB to 1 L of deionized water in 2 L Pyrex flask (see Note 21).

37. Autoclave on a liquid cycle to sterilize media (121 °C for 20 min).

38. Allow to cool to 50 °C, and add 1 mL (50mg/mL) kanamycin to the sterilized 1 L LB media.

3.4.2. Overexpressing Proteins in Cell Cultures. (Time of Completion: 1 day)

39. Pipet 5 mL LB kanamycin into sterilized 15 mL Falcon tubes for each variant protein that will be expressed.

40. Add 2 µL of bacterial glycerol stock into corresponding falcon tubes (see Note 22).

41. Incubate overnight in shaker for 16 h at 37 °C and 220 rpm.

42. Add 5 mL overnight culture to autoclaved 1 L LB kanamycin media.

43. Incubate at 37 °C and shake at 220 rpm until the optical density of 0.6 at 600 nm (OD600), measured with a UV-Vis Spectrometer, is reached (see Note 23, 24).

44. Incubate at 25 °C with shaking at 220 rpm for 16 h.

3.4.3. Harvesting Cells. (Time of Completion: 2–3 h)

46. Distribute the 1 L cell culture into centrifuge bottles.

47. Spin down at RCF of 4,400 × g for 10 min at 4 °C in a Thermo Scientific Sorvall RC 6 Plus Superspeed centrifuge (see Note 25).

48. Discard supernatant and resuspend cell pellet with 30 mL lysis buffer (50mM NaH_2PO_4, 500mM NaCl, pH 8.0) (see Note 26).

49. Transfer resuspended samples to 50 mL Corning tubes. Here, the samples can be stored at -20 °C if needed for up to 48 h, but proceeding to cell lysis and protein purification (see Subheading 3.4.4) on the same day is preferred.

3.4.4. Cell Lysis and Protein Purification. (Time of Completion: 1 day)

50. Lyse cells in homogenizer.

51. Transfer the cell lysate into 50 mL centrifuge tubes and spin down at RCF of 26,900 × g for 45 min.

52. Prepare Ni-NTA columns for purification by running two full column volumes of deionized water through the nickel-chelated resin, followed by equilibration with a full column volume of lysis buffer.

53. Load supernatant onto Ni-NTA columns (see Note 27).

54. Wash column with 50 mL wash buffer (50mM NaH2PO4, 500mM NaCl, 20 mM imidazole, pH 8.0) to remove non-His tagged proteins.

55. Elute His tagged protein with 5 mL elution buffer (50mM NaH2PO4, 500mM NaCl, 250 mM imidazole, pH 8.0) (see Note 28).

56. Add 150 µL of 100 µM ubiquitin-like-specific-protease 1 (Ulp1) (see Note 29).

57. =Allow 8–14 h for Ulp1 to cleave His6-SUMO fusion proteins at 4 °C.

58. Dilute the solution to 25 mM imidazole by adding lysis buffer to the sample.

59. Clean Ni-NTA columns with two column volumes of DI water, followed by two bed volumes of 0.2 µm-filtered solution of 6 M guanidine hydrochloride (GdHCl) in 0.2 M acetic acid. Then wash with another two column volumes of DI water.

60. Pass Ulp1-treated protein samples through clean Ni-NTA columns, and collect the flow-through in a clean, labeled corning tube (see Note 30).

61. Repeat steps 59 & 60 to ensure that protein sample has been sufficiently separated from His6-SUMO tags (see Note 31).

62. Concentrate purified samples to 6 mL for subsequent FPLC injection (see Subheading 3.4.5) in an Amicon Stirred Cell concentrator using a 10 kDa molecular weight cut-off (MWCO) membrane.

3.4.5. Fast Protein Liquid Chromatography. (Time of Completion: 2–3 h)

63. Prepare mobile phase buffer in which the purified protein will be stored (20 mM Tris, 150 mM NaCl, pH 8, or pH 7.0 if the protein will be fluorescently labeled) (see Note 32).

64. Divide the 6 mL of concentrated protein sample into 1 mL aliquots in six 1.5 mL centrifuge tubes and spin down at RCF of 18,000 × g for 2 min on a benchtop centrifuge. This will allow for removal of any precipitate and particles from the sample before FPLC.

65. Draw the supernatant from the centrifuge tubes into a 10 mL syringe using a long blunt-end needle.

66. Exchange the needle on the syringe with an adaptor for connecting to the FPLC sample injection port.

67. Remove all bubbles from the syringe.

68. Attach the syringe to the sample injection port on the FPLC. Do so without introducing air into the instrument by filling the injection port with DI water as the syringe adaptor is inserted.

69. Run the FPLC with HiLoad 16/600 Superdex 75 size-exclusion column, at 0.8 mL/min flow rate, and 0.5 MPa maximum pressure.

70. Collect eluate peak fractions for proteins of interest.

71. Following purification, concentrate eluate to desired concentration with an Amicon Stirred Cell concentrator using a 10 kDa molecular weight cut-off (MWCO) membrane.

3.5. Fluorophore Labeling of Clock Protein. (Time of Completion: 1 day)

72. Mix thiol-reactive fluorescent dye (1 mg 6IAF resuspended in 80 uL methanol) with protein at the ratio recommended for the particular dye being used (see Note 33).

73. Incubate at 4 °C for 14–16 h, covered to avoid light contamination.

74. Use Amicon Stirred Cell concentrator with 10 kDa MWCO membrane to concentrate sample to 2 mL.

75. Split the 2 mL sample into two 1.5 mL centrifuge tubes and spin down at RCF of 18,000 × g for 2 min using a benchtop centrifuge.

76. Repeat steps 65–68.

77. Run the FPLC with HiPrep 26/10 Desalting Column, 3 mL/min flow rate, and 0.3 MPa max pressure to separate labeled protein from free fluorophore.

78. Collect eluate from 11–19 mL.

79. Following purification, concentrate eluate to desired molarity with the Amicon Stirred Cell concentrator and 10 kDa MWCO membrane.

3.6. Fluorescence Anisotropy Measurements. (Time of Completion: 1–2 h)

80. Add reaction buffer (20 mM Tris, 150 mM NaCl, 5 mM MgCl2, 1 mM ATP, 0.5 mM EDTA, 50 µg/mL kanamycin, pH 8.0) to 1.5 mL centrifuge tubes.

81. Add KaiA to a final concentration of 1.2 µM.

82. Add unlabeled KaiB-FLAG to a final concentration of 3.45 µM.

83. Adjust ATP concentration to 1 mM using ATP stock solution.

84. Adjust MgCl2 concentration to 5 mM.

85. Add FLAG-KaiC to a final concentration of 3.5 µM.

86. Start 30-min timer, which indicates when to begin fluorimeter data collection (see Note 34).

87. Load clock reaction samples into 1 mL syringe, attach 0.2 µm membrane filter, and filter samples into fresh 1.5 mL centrifuge tubes (see Note 35).

88. Transfer into a fresh 1.5 mL centrifuge tube the volume of sample necessary to achieve a final volume of 600 µL upon adding fluorescently labeled protein in step 89.

89. In the dark, add KaiB-FLAG-K25C-fluorophore to a final concentration of 0.05 µM and total volume of 600 µL.

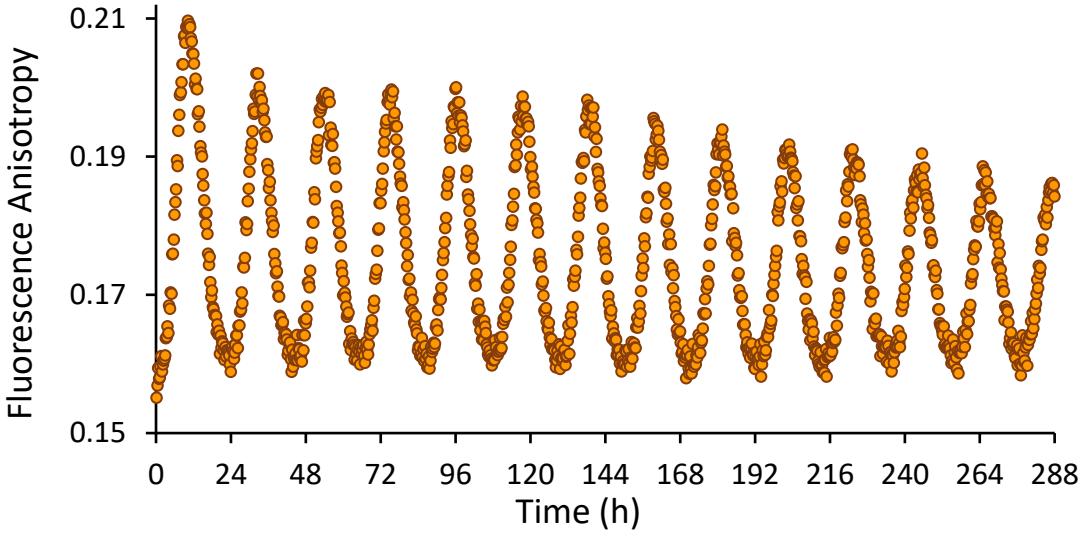
90. In the dark, mix the sample and transfer it to a fluorimeter cuvette.

91. Place the cuvette in the fluorimeter and start data collection once the 30-min timer (see step 86) expires. Record the time representing t=0.

4. Expected Results

Appendix B Fig.5 shows real-time fluorescence anisotropy measurements of KaiB-FLAG-K25C-fluorophore over the course of 12 days. Note that robustness of the oscillation indicates that the experiment could have been extended considerably longer. Trough anisotropies are when labeled KaiB is mostly free in solution. In contrast, peak anisotropies are when KaiB is bound to KaiC and KaiA. The stability of the troughs is likely due to the stability of labeled KaiB. The slow decrease of peak anisotropies is likely due to a steady decrease in ATP and functional KaiC protein levels. In order to minimize potential artifacts due to the fluorophore, only 1.4% (50 nM) KaiB was labeled. Fluorescence intensities over 12 days decreased from 720,000 counts to 670,000, indicating that photobleaching is not a significant concern. A significant advantage of this protocol is that it allows hundreds of *in vitro* reactions to be monitored simultaneously in real time using a plate reader with minutes time resolution for weeks (this protocol has been employed to monitor oscillations on the Tecan

Spark 10M and BMG CLARIOstar plate readers with nearly identical results relative to data collected on the PC1). Assuming that 15 ATP molecules are consumed per day per KaiC particle,[64] after 12 days the ATP concentration is expected to be 0.3–0.4 mM. Note that the oscillator begins to fail around 0.1 mM ATP.[64] Although only KaiB was labeled in this protocol, KaiA and KaiC can in principle also be fluorescently labeled, although care must be taken regarding their naturally occurring cysteinyl residues.



Appendix B Fig.5. Fluorescence anisotropy of 0.05 μM KaiB-FLAG-K25C-6IAF in a reaction also containing unlabeled KaiA, KaiB- FLAG,[84] and FLAG-KaiC at 1.2 μM, 3.45 μM, and 3.5 μM, respectively. Data points were collected every 15 min for 12 days.

5. Notes

1. We used the pET28b+ bacterial expression vector, which produces N-His6-SUMO-proteins. Kanamycin resistance is important for several steps outlined in this protocol. If other vectors are used, adjustments to the antibiotic steps throughout this protocol may be needed.

2. Quikchange PCR primers for Synechococcus elongatus KaiB-FLAG-K25C:

Forward-CCAAACTCAGTCCGTGCCCTCTGCACGCTCAAGAACATTCTCG

Reverse-CGAGAATGTTCTTGAGCGTGCAGAGGGCACGGACTGAGTTTGG

3. Autoclave 1 L of Luria broth (LB) media broth in 2 L Erlenmeyer flask on liquid cycle. Allow to cool to 50 °C and add 1 mL (50mg/mL) kanamycin to the sterilized 2 L LB flask. Pour into sterile plastic plates and immediately cover with lids. Let sit at room temperature for 20 min before wrapping in aluminum foil and storing at 4 °C until use. We find that these plates are good for up to one month.

4. Lysis buffer, wash buffer, and elution buffers are best stored at 4 °C until use. Elution buffer should avoid light, because the high concentration of imidazole turns the solution yellow with light exposure. However, slight discoloration does not affect the elution of His-tag proteins.

5. Proteins can crosslink through cysteinyl –SH groups. To minimize this unwanted reaction, a final concentration 20 mM TCEP was added to the elution volume following Ni-NTA column purification. More TCEP can be added after the 2nd Ni-NTA column purification step, preceding concentration of the sample for FPLC (see steps 55–62). No additional TCEP is added following size-exclusion chromatography as to not interfere with 6IAF labeling efficiency.

6. Here, for each fluorescently labeled protein preparation, 1 mg of 6IAF was suspended with 80 µL of methanol and then mixed in a 5:1 ratio with KaiB-FLAG-K25C at pH 7.0 to facilitate conjugation (see step 72).

7. After labeling, a desalting column is used to both adjust pH to 8.0 for oscillation reactions while also removing free dye from the sample.

8. Here, we fused N- and C-terminal FLAG-tags (DYKDDDDK) to KaiC and KaiB proteins, respectively, to increase solubility and stability.

9. An Avestin C3 homogenizer was used to produce homogenous cell-disrupted samples. Allowing the samples to cycle through the instrument for ~5 min was sufficient to consistently achieve homogenous products. Subsequent centrifugation yielded clear separation of supernatant containing soluble proteins and cell debris pellet.

10. Protein concentrations were determined with bovine serum albumin (BSA) standard curve Bradford Assay. A standard curve was made fresh each time a protein was measured.

11. Simply wrapping the corning tubes with aluminum foil, fixed with tape or parafilm is sufficient to protect the fluorescent dye from light exposure during labeling.

12. The concentrator employed here was Amicon stirred cell 50 mL concentrator with a 10 kDa molecular weight cutoff regenerated cellulose membrane. This apparatus was connected to a nitrogen gas line to provide

pressure, and magnetic stir plate allowed for stirring while concentrating. Concentration occurred in a 4 °C refrigerator.

13. ISS Photon Counting Spectrofluorimeter (PC1) shown in Appendix B Fig.3, with 300 W xenon arc lamp attenuated such that fluorescently-labeled samples yield 600,000–800,000 photon counts, was used for all fluorescence measurements described here. This steady-state fluorimeter was setup with a three-stage cuvette system and water bath to control sample temperature. Excitation and emission wavelengths are easily manipulated, allowing for measurements of multiple fluorescent dyes simultaneously.

14. ATP and MgCl2 stocks at concentrations of 300 mM and 200 mM, respectively, were used. These should be prepared in 20 mM Tris with pH 8.0. Note that KaiA and KaiB protein stocks lack ATP and MgCl2.

15. The PCR products should be run on 1.5% agarose gels before performing DpnI digestion. Load 3 µL PCR product and 3 µL 6x orange loading dye. Mix well with micropipette and heat to 100 °C for 1 minute before loading agarose gel. Only add DpnI to the products that show bright bands at the correct size of the plasmid.

16. Sample purity can be obtained by comparing the absorbance at 260 nm against that of 280 nm (A260/280). Highly pure DNA in should be ~1.8, while highly pure RNA should be ~2.0. Proteins absorb light at 280 nm. Therefore, lower ratios are indicative of protein contamination in the sample.[161]

17. For the final five minutes of the 30 min period in which the Quikchange PCR product is on ice, place the 2x YT media broth stock tubes in the hot water bath at 42 °C. Remove the 2× YT media broth from the hot water bath at the same time the heat shock ends (see step 11).

18. Glass rods should be sterilized prior to spreading cells on LB-kan+ agar plates. Flame the glass rod for 3–5 s, dip into ethanol, flame again for 3–5 s, and then allow cooling for 10–12 s. Repeat this process between streaking samples.

19. To confirm that transformation was successful, pick a single colony from the transformation plate and inoculate in 5 mL LB-kan+ media broth. After overnight incubation at 37 °C with shaking at 220 rpm, use a miniprep kit to isolate DNA. DNA sequencing of this sample will determine whether the Quikchange PCR and transformation steps were successful (see Subheading 3.3).

20. Following incubation, 1 µL incubated transformation product will be used in step 24, and the remained of the cultured 5 mL LB-kan+ broth media may be stored at 4 °C. This culture will be utilized following gel electrophoresis analysis to ensure proper DNA length (see step 29).

21. LB media should be used for KaiA and KaiB-FLAG expression. For FLAG-KaiC, M9 minimal media is highly recommended. To produce 1 L of M9 media, mix 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1 g NH_4Cl, then autoclave on liquid cycle. Following sterilization, add 10 mL (0.2 µm filtered) 20% D-glucose, 2 mL 1 M $MgSO_4$, 100 µL 1 M $CaCl_2$, and 1 mL 50 µg/mL kanamycin.

22. All glycerol stocks consist of transformed cells in 16% glycerol. These solutions are prepared by adding 100 µL of 80% glycerol (autoclaved) to 400 µL of transformed cell media. These samples are stored at −80 °C until use.

23. After 4–6 h of incubation, optical density measurements of cell cultures at OD_{600nm} are carried out on a Hach DR 6000EDU UV-Vis spectrophotometer, with DI H_2O used as a blank.

24. IPTG induces protein expression in transformed E. coli by binding to lac operon repressor. This binding causes the repressor to dissociate from the DNA promoter region.

25. For temperature controlled high speed/large volume centrifugation, a Thermo Scientific Sorvall RC 6 Plus Superspeed centrifuge with FiberLite F10-6x500y (500 mL bottles) and Sorvall Instruments SS-34 (50 mL bottles) rotors are used. All samples must be within 0.1 g of one another to ensure the rotors are balanced (steps 47 & 51).

26. When preparing lysis buffer for cell pellet resuspension of FLAG-KaiC expressing cells, a final concentration of 1 mM ATP must be added to the buffer. FLAG-KaiC is a homohexameric protein with ATP molecules bound at protomer-protomer interfaces. Without ATP present KaiC is unstable and aggregates, reducing yield. ATP must also be present in the lysis buffer during cell cracking, in the nickel column wash and elution buffers, and in the mobile phase buffer during FPLC purification.

27. Preceding the SUMO tag is a 6-His-tag that binds the Ni^{2+} ions, trapping the proteins of interest on the Ni-NTA column.

28. It is advised that 20 µL aliquots of supernatant, wash, and eluate be collected and analyzed by SDS-PAGE following Ni-NTA purification. This can help to quickly determine whether the protein of interest is in the eluate (desirable) or wash (undesirable).

29. The Ulp1 enzyme specifically cleaves His6-SUMO tag from the fusion protein, allowing for purification by subsequent passes through Ni-NTA columns in which the His6-SUMO tag binds while the protein does not.

30. When passing Ulp1-treated protein samples through Ni-NTA columns, it is important to regulate flow rates to ensure that the free His6-SUMO fusion tag can

be capt6xured on the Ni-NTA column, while proteins of interest pass through. This was performed by attaching a 1–2” section of soft rubber tubing to the bottom of each Ni-NTA column, which can then be partially clamped to allow flow rates of ~50–60 drop/min.

31. After cleavage of His6-SUMO by Ulp1 and purification by Ni-NTA columns, a confirmation step can be performed to verify that the protein of interest is present and pure. Transfer 3–5 µL of nickel column eluate to a centrifuge tube and add an equal volume of 2× SDS-dye. Load the sample alongside a protein ladder on SDS polyacrylamide gel (17% for KaiB) and run for 30 min at 60 V, followed by 100 min at 120 V. A comparison of stained bands against molecular weight markers allow for the estimation of protein purity and yield.

32. The preferred ratio of 6IAF to protein is 10–20:1, although we have had success at 5:1 fluorescently labeling KaiB. During the conjugation reaction the recommended pH is 7.0–7.5, which is accomplished by preparing the mobile phase for FPLC at this pH. All labeling was performed at 4 °C overnight in the dark.

33. 6IAF was chosen because it is inexpensive with high quantum yield, photostability, and moderate excitation/emission spectrum such that it may be paired as a FRET-acceptor or -donor in the future. As stated in step 63, a 5:1 ratio of 6IAF to protein is sufficient for ~99% labeling efficiency (by mass spectrometry, data not shown).

34. During the 30-min timer duration, prepare the fluorimeter for data collection. Here, using 6IAF as a reporter, the monochromators on the PC1 excitation and emission wavelengths of 492 nm (slit width 2.0 nm) and 520 nm (slit width 1.0 nm), respectively, were read every 15 minutes. Fluorescence anisotropy measurements are achieved on the PC1 with an emission polarizer isolating parallel emission detection followed by subsequent perpendicular emission detection with the same photomultiplier tube (PMT). G-factor determination is set to the “once” option to adjust parallel and perpendicular gain values in order to eliminate instrumental bias in fluorescence anisotropy measurements.

35. Filtering the solutions with low protein-binding 0.2 µm membrane ensures bacteria and/or large contaminants are not present in the final reaction mixtures. This step may be omitted if long term oscillations, many days/weeks, are not planned.

Author Contributions: Conceptualization, J.H. and A.L.; Methodology, J.H. and A.L.; Formal Analysis, J.H.; Writing--Original Draft Preparation, J.H, and A.L.; Writing--Review & Editing, J.H, A.C., and A.L.; Supervision, A.C., Y.G.C, and A.L.; Funding Acquisition, A.L.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix C

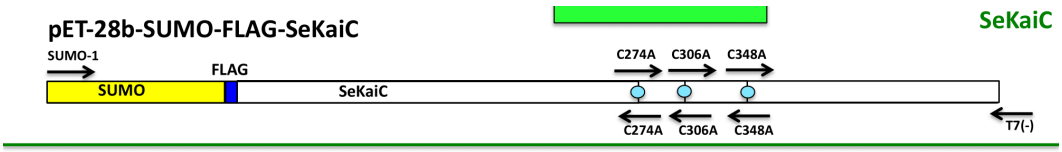
Multistep PCR reactions for fluorophore labeling constructs of KaiC, KaiA, SasA, CikA, and RpaA

All gene sequences have been adjusted to scale, with 1 “corresponding to a specified number of base pairs (bps) at the beginning of each multistep PCR workflow.

Mutation sites are designated by light blue circles with mutations made shown with red stars. Primes are represented by black arrows pointing to the right (forward primers) or to the left (reverse primers).

Intermediate and final products are shown in brackets.

KaiC constructs



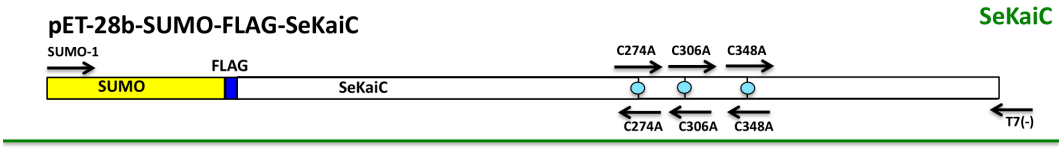
Scale:
1” = ~216 bp

Targets:

#1. (C) pET-28b-SUMO-FLAG-SeKaiC-**C274A**-**C306A**-**C348A**

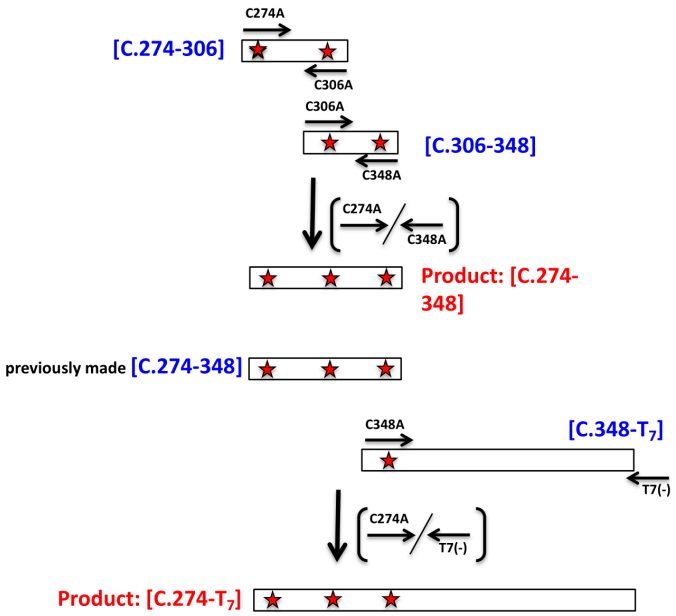
#2. (Ce) pET-28b-SUMO-FLAG-SeKaiC-**E128C**-C274A-C306A-C348A

#3. (Cc) pET-28b-SUMO-FLAG-SeKaiC-C274A-C306A-C348A-**Cys**

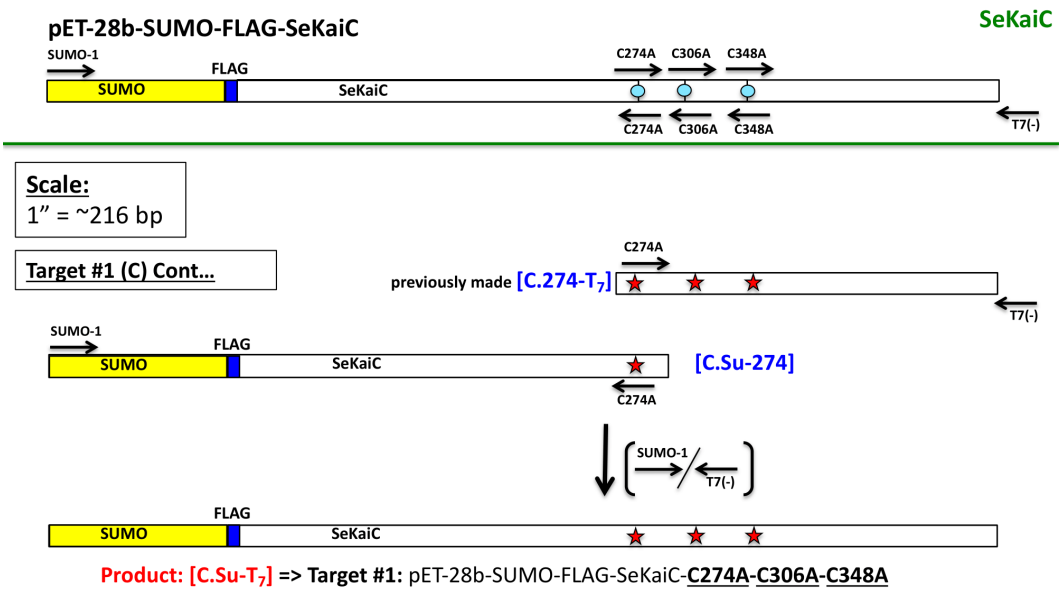


Scale:
1” = ~216 bp

Target #1 (C)

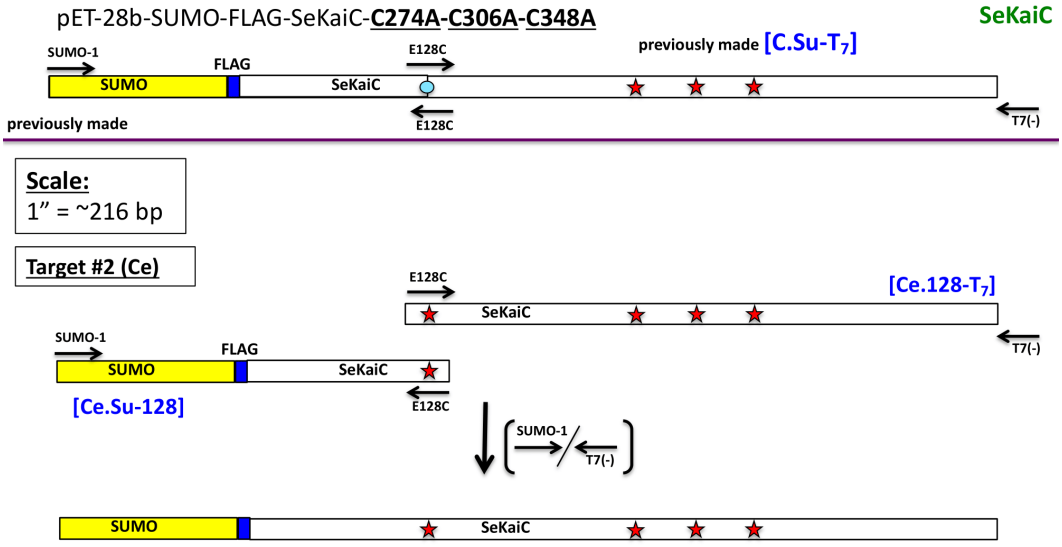


#1. (C) pET-28b-SUMO-FLAG-SeKaiC-**C274A**-**C306A**-**C348A**



Product: [C.Su-T_7] => Target #1: pET-28b-SUMO-FLAG-SeKaiC-**C274A-C306A-C348A**

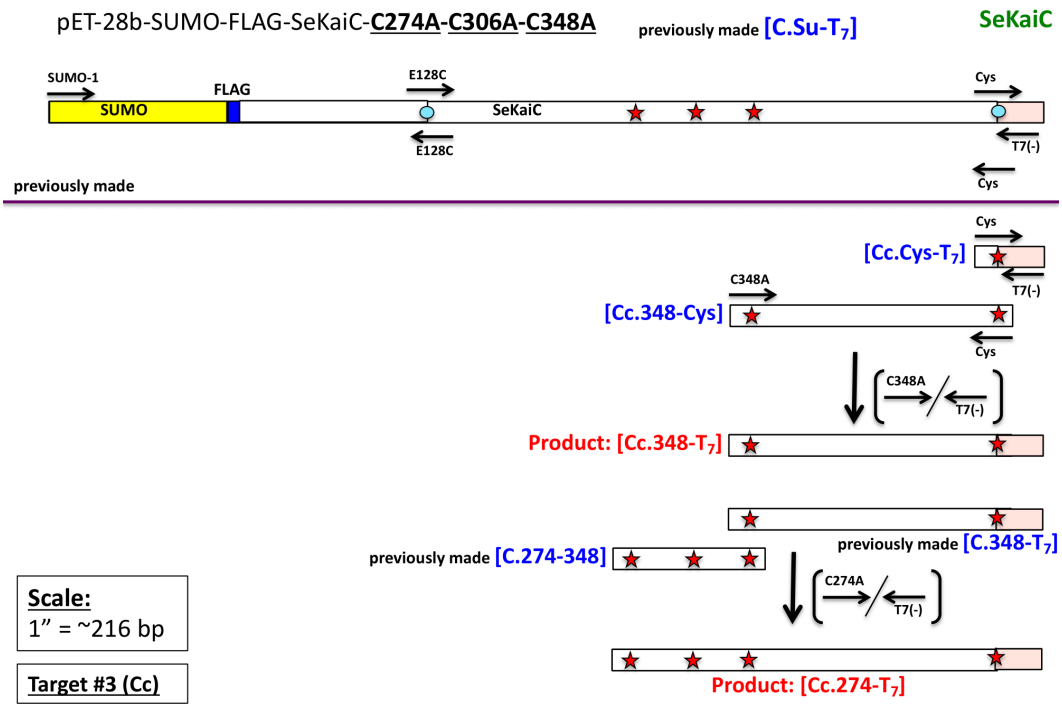
#1. (C) pET-28b-SUMO-FLAG-SeKaiC-**C274A-C306A-C348A**



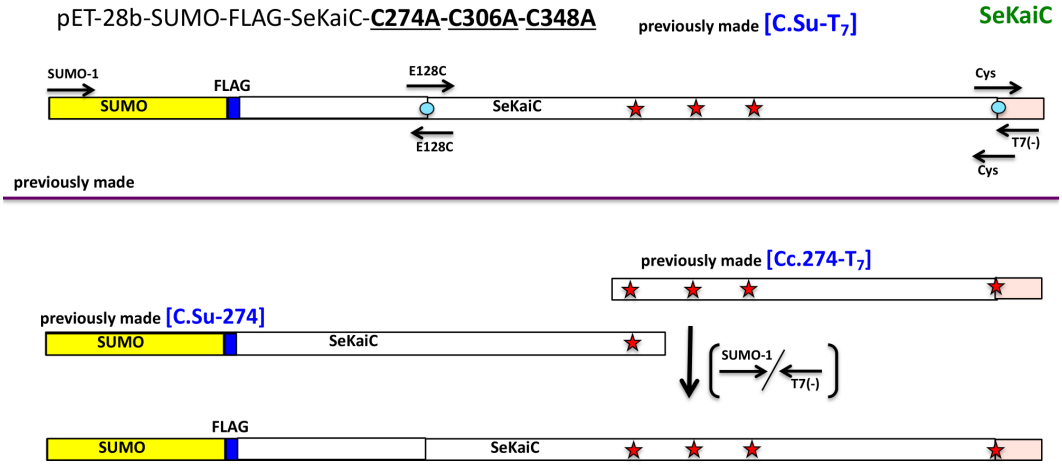
Product: [Ce.Su-T_7] => Target #2: pET-28b-SUMO-FLAG-SeKaiC-**E128C**-C274A-C306A-C348A

Note: May need larger overlap, which would mean creating [C.128-306] and [C.Su-128] first, to overlap and extend to Product: [C.Su-306] which would provide much more overlap region for the full gene synthesis

#2. (Ce) pET-28b-SUMO-FLAG-SeKaiC-**E128C**-C274A-C306A-C348A



#3. (Cc) pET-28b-SUMO-FLAG-SeKaiC-C274A-C306A-C348A-Cys



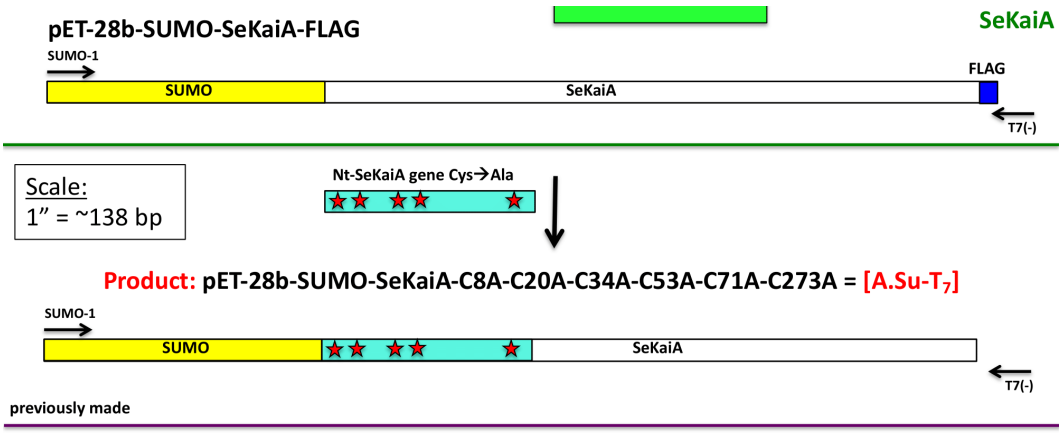
Product: [Cc.Su-T_7] => Target #3: pET-28b-SUMO-FLAG-SeKaiC-C274A-C306A-C348A-Cys

Scale:
1" = ~216 bp

Target #3 (Cc) Cont…

#3. (Cc) pET-28b-SUMO-FLAG-SeKaiC-C274A-C306A-C348A-Cys

KaiA constructs



Targets:

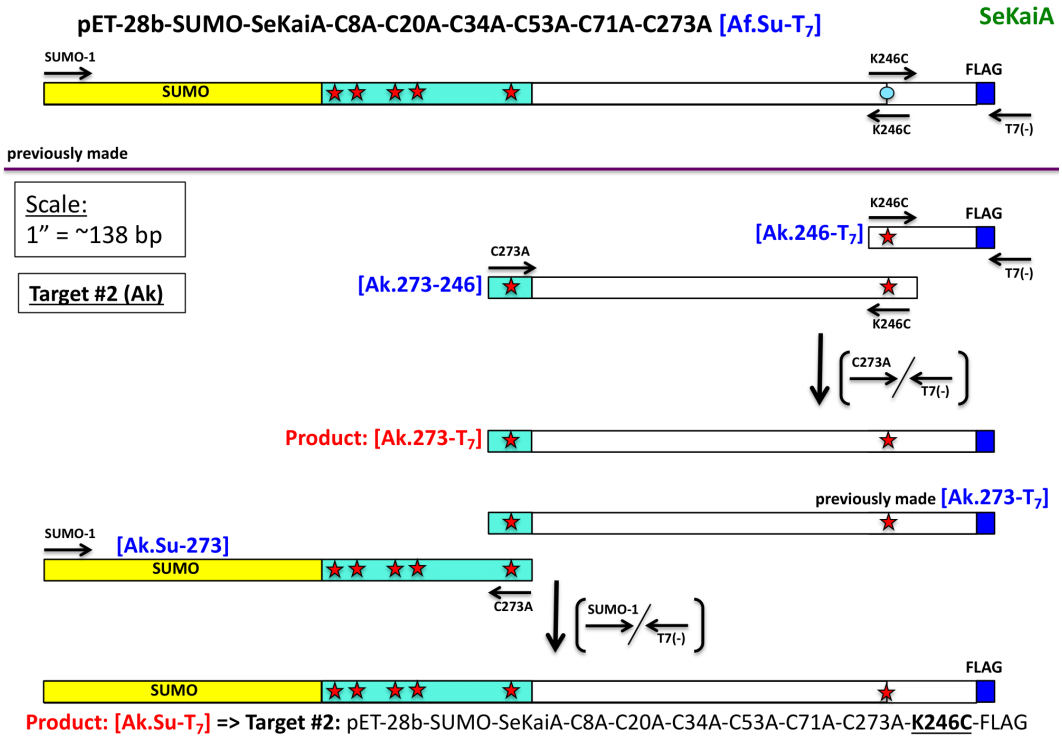
#1. (Af) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**FLAG**

#2. (Ak) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**K246C**-FLAG

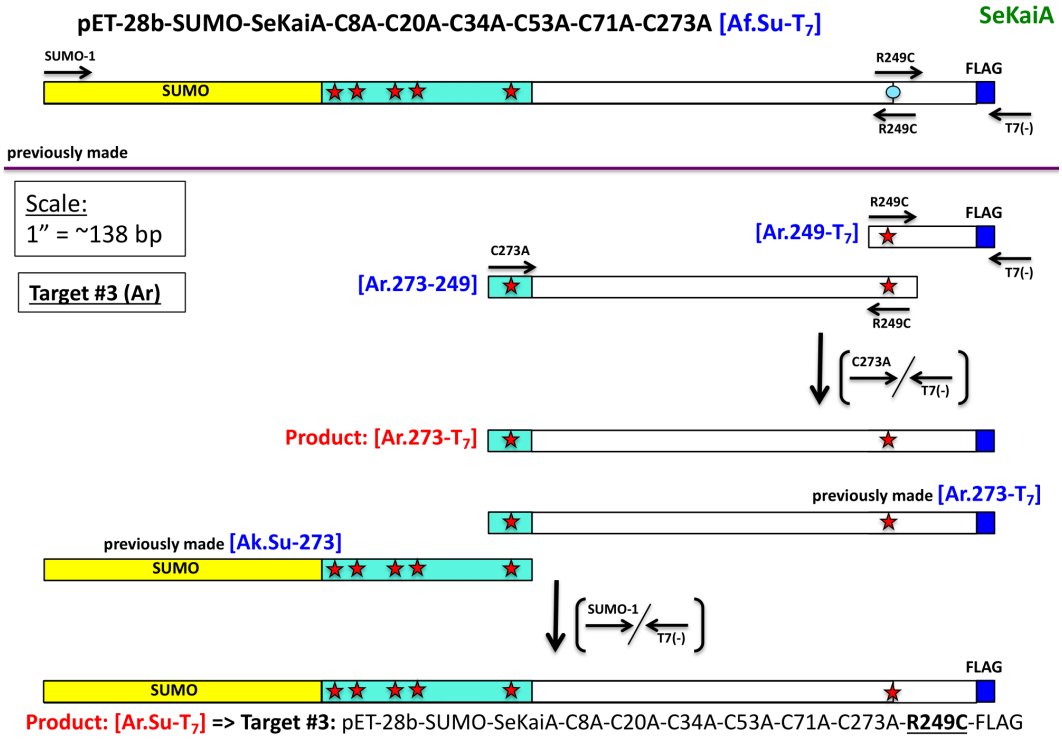
#3. (Ar) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**R249C**-FLAG

Product: [Af.Su-T7] => Target #1: pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**FLAG**

#1. (Af) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**FLAG**

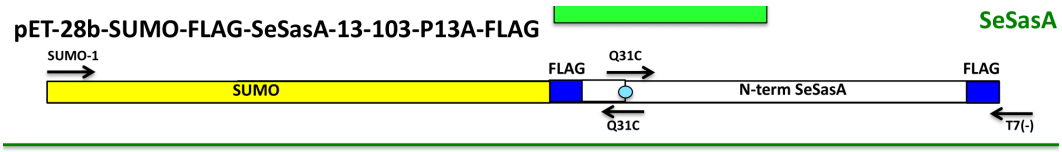


#2. (Ak) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-C273A-**K246C**-FLAG



#3.) pET-28b-SUMO-SeKaiA-C8A-C20A-C34A-C53A-C71A-**R249C**-FLAG

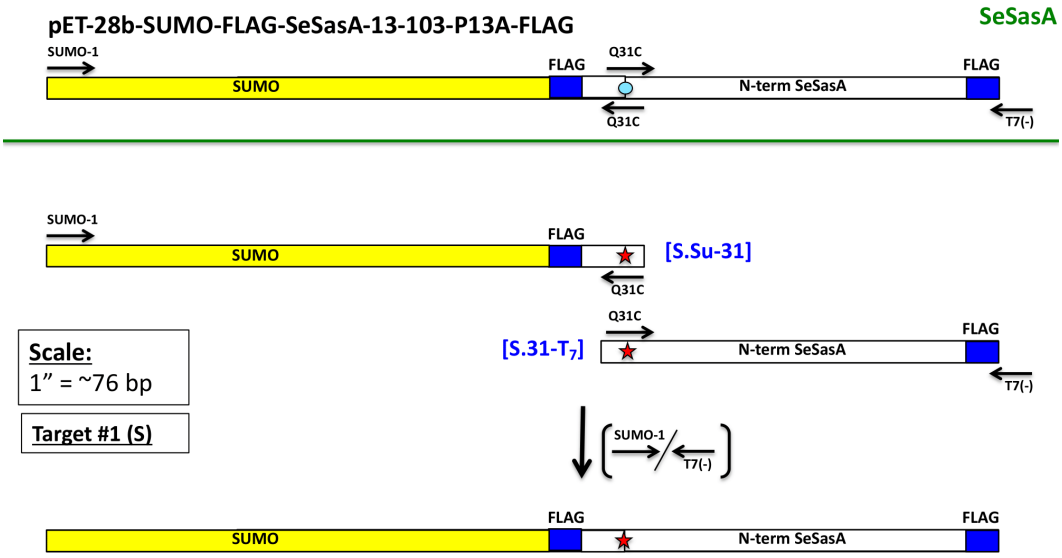
SasA constructs



Scale:
1” = ~76 bp

Targets:

#1. (S) pET-28b-SUMO-FLAG-SeSasA-13-103-P13A-**Q31C**-FLAG

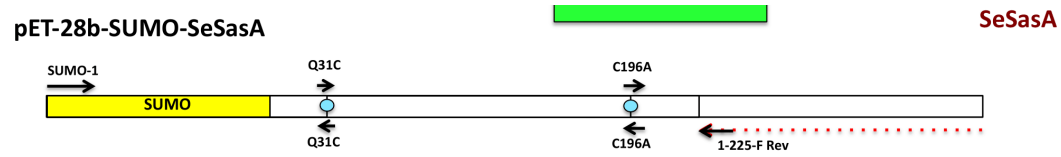


Scale:
1” = ~76 bp

Target #1 (S)

Product: [S.Su-T_7] => Target #1: pET-28b-SUMO-FLAG-SeSasA-13-103-P13A-**Q31C**-FLAG

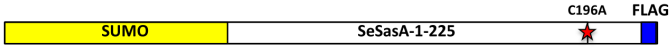
#1. (S) pET-28b-SUMO-FLAG-SeSasA-13-103-P13A-**Q31C**-FLAG



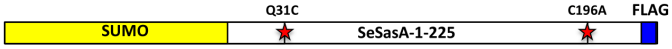
Scale:
1” = ~172.33 bp

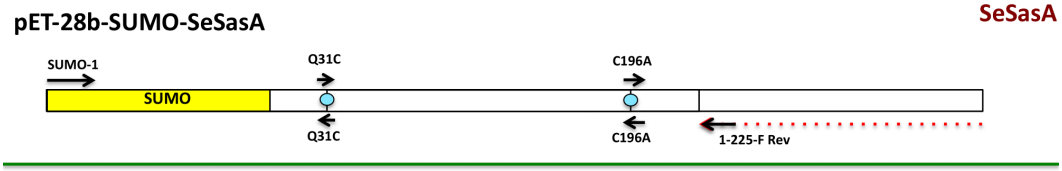
Targets:

#1. (Sf.) pET-28b-SUMO-SeSasA-**1-225**-**FLAG**



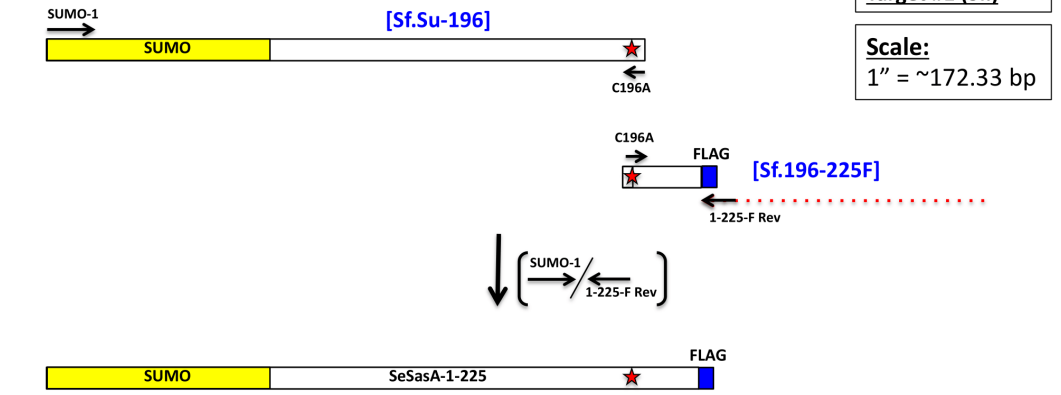
#2. (Sfq.) pET-28b-SUMO-SeSasA-**1-225**-**Q31C**-**FLAG**





Target #1 (Sf.)

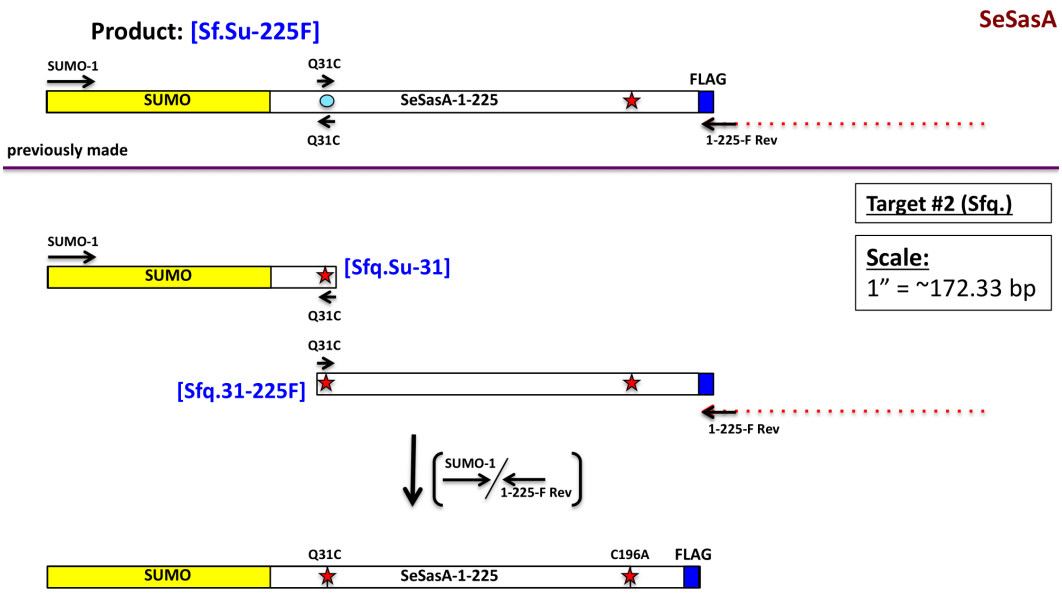
Scale:
1” = ~172.33 bp



Product: [Sf.Su-225F] => Target #1: pET-28b-SUMO-SeSasA-**1-225**-**C196A**-**FLAG**

#1. (Sf) pET-28b-SUMO-SeSasA-**1-225**-**C196A**-**FLAG**

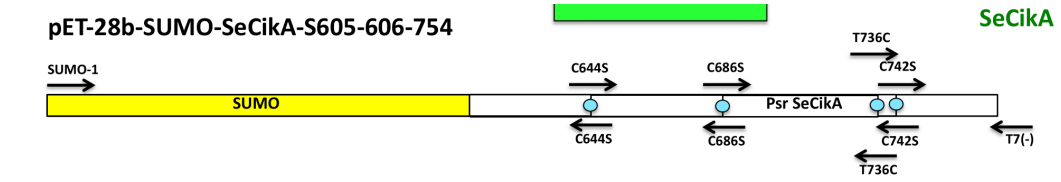
SeSasA



Product: [Sfq.Su-225F] => Target #2: pET-28b-SUMO-SeSasA-1-225-**Q31C**-C196A-FLAG

#2. (Sfq) pET-28b-SUMO-SeSasA-1-225-**Q31C**-C196A-FLAG

CikA constructs

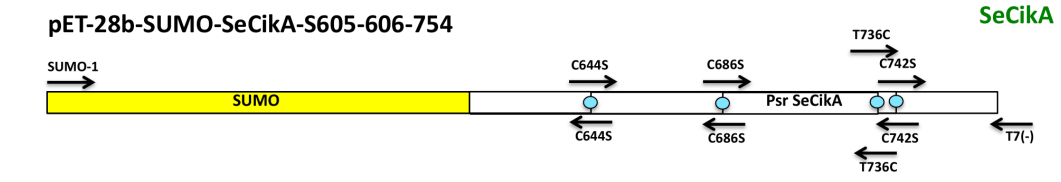


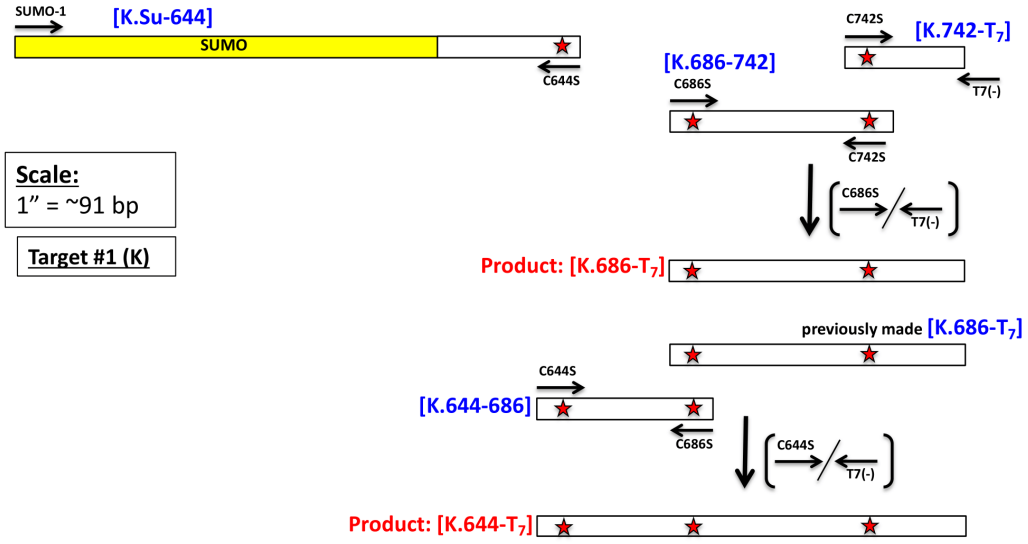
Scale:
1" = ~91 bp

Targets:

#1. (K) pET-28b-SUMO-SeCikA-S605-606-754-**C644S**-**C686S**-**C742S**

#2. (Kt) pET-28b-SUMO-SeCikA-S605-606-754-**C644S**-**C686S**-**C742S**-**T736C**

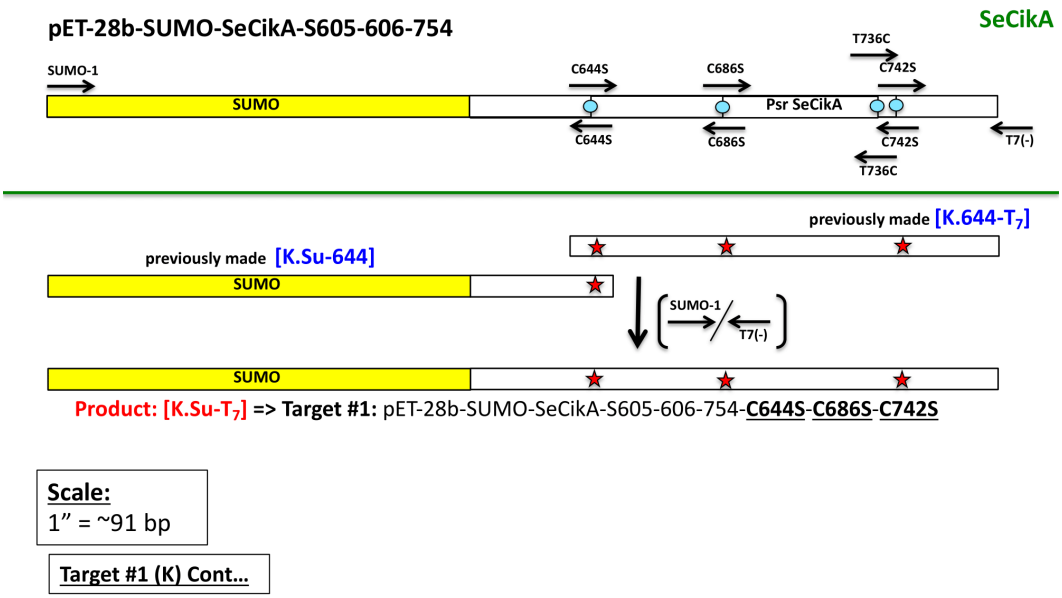




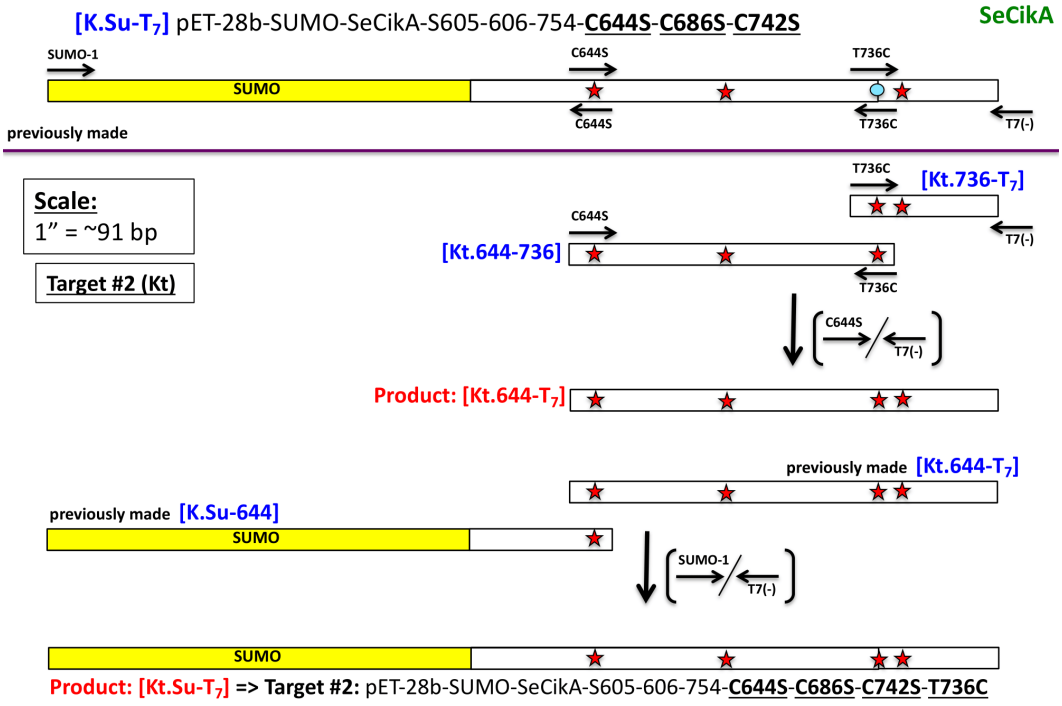
Scale:
1" = ~91 bp

Target #1 (K)

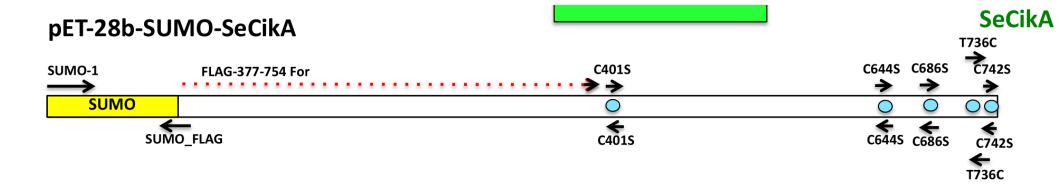
#1. (K) pET-28b-SUMO-SeCikA-S605-606-754-**C644S**-**C686S**-**C742S**



#1. (K) pET-28b-SUMO-SeCikA-S605-606-754-C644S-C686S-C742S



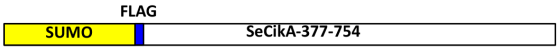
#2. (Kt) pET-28b-SUMO-SeCikA-S605-606-754-C644S-C686S-C742S-T736C



Targets:

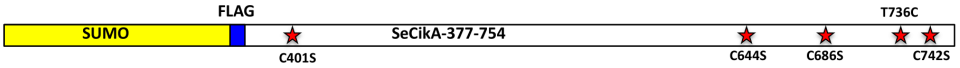
#1. (Kf.) pET-28b-SUMO-**FLAG**-SeCikA-**377-754**

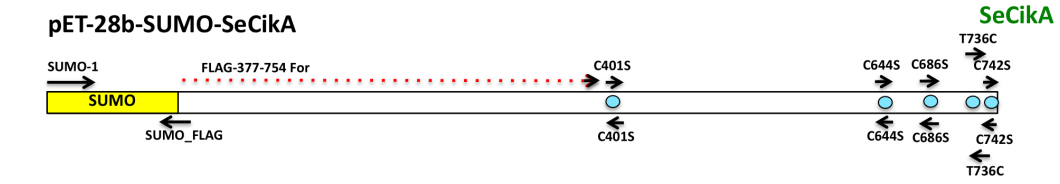
Scale:
1" = ~292 bp

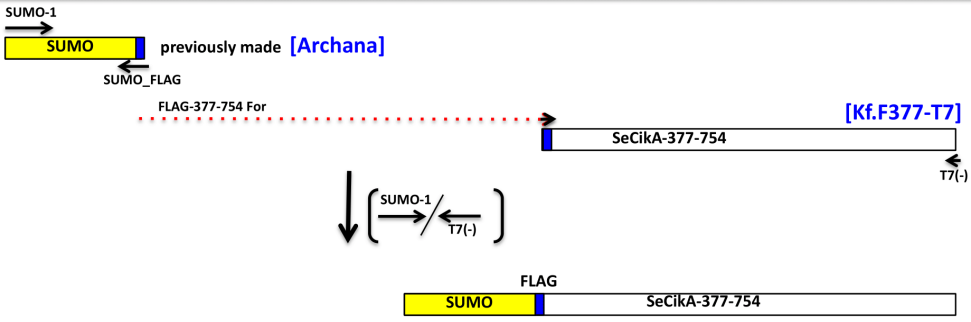


#2. (Kc.) pET-28b-SUMO-FLAG-SeCikA-377-754-**C401S**-**C644S**-**C686S**-**C742S**-**T736C**

Scale:
1" = ~169 bp





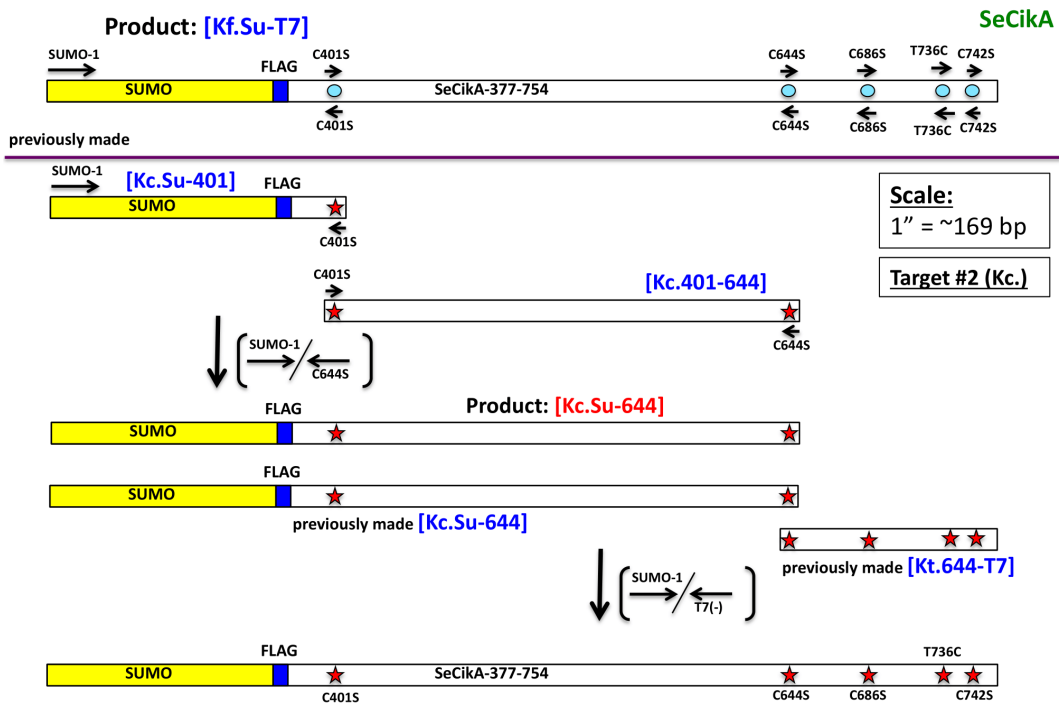


Product: [Kf.Su-T7] => Target #1: pET-28b-SUMO-**FLAG**-SeCikA-**377-754**

Scale:
1" = ~292 bp

Target #1 (Kf.)

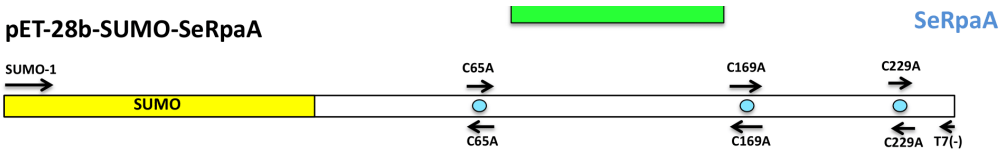
#1. (Kf.) pET-28b-SUMO-**FLAG**-SeCikA-**377-754**



Product: [Kc.Su-T7] => Target #2: pET-28b-SUMO-FLAG-SeCikA-377-754-**C401S**-**C644S**-**C686S**-**C742S**-**T736C**

#2. (Kc.) pET-28b-SUMO-FLAG-SeCikA-377-754-**C401S**-**C644S**-**C686S**-**C742S**-**T736C**

RpaA constructs



Scale:
1” = ~123.67 bp

Targets:

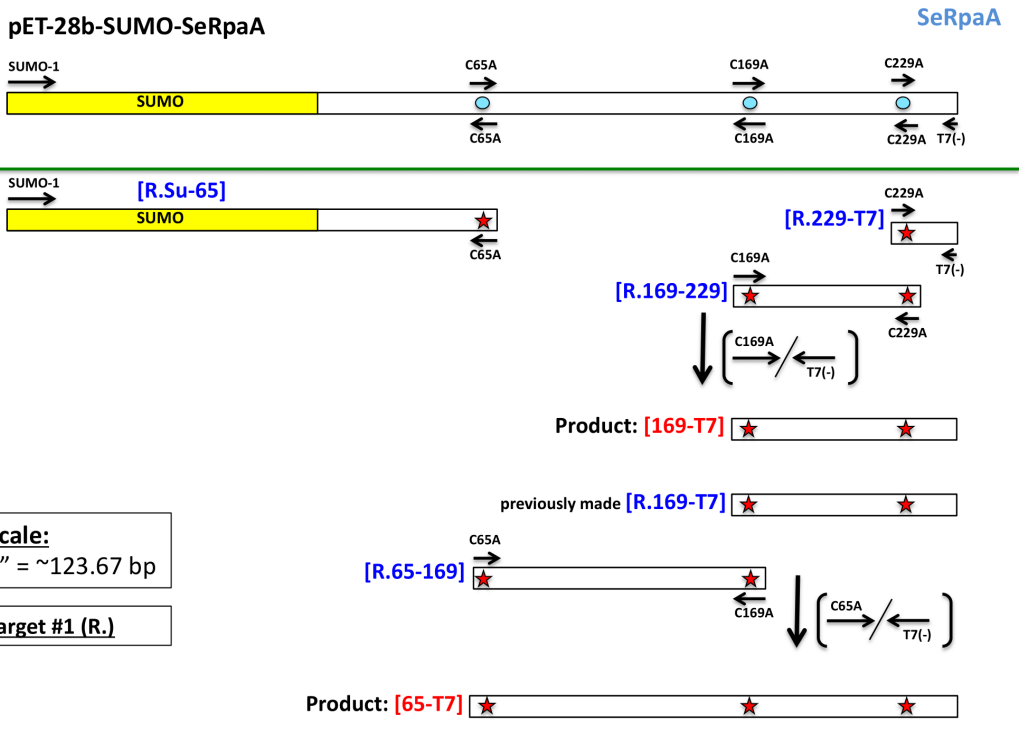
#1. (R.) pET-28b-SUMO-SeRpaA-**C65A**-**C169A**-**C229A**

#2. (Rs.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**S12C**

#3. (Re.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**E16C**

#4. (Ri.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**I36C**

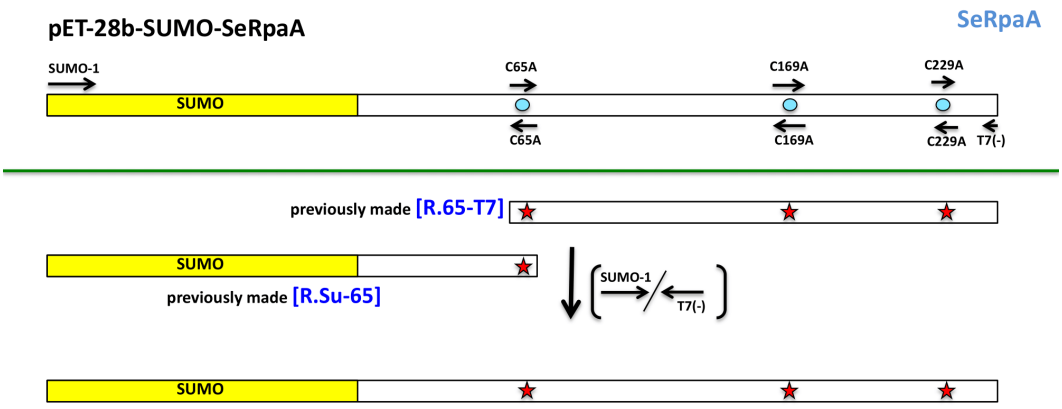
#5. (Rt.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**T63C**



Scale:
1” = ~123.67 bp

Target #1 (R.)

#1. (R.) pET-28b-SUMO-SeRpaA-**C65A**-**C169A**-**C229A**

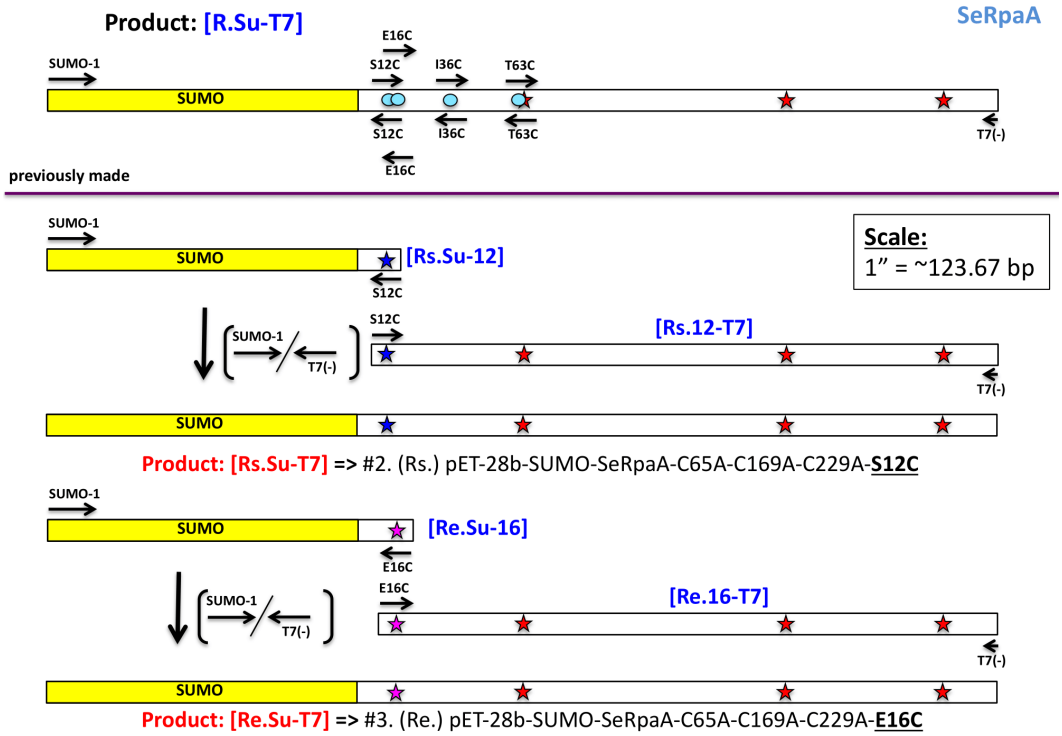


Product: [R.Su-T7] => #1. (R.) pET-28b-SUMO-SeRpaA-**C65A**-**C169A**-**C229A**

Scale:
1" = ~123.67 bp

Target #1 (R.) Cont…

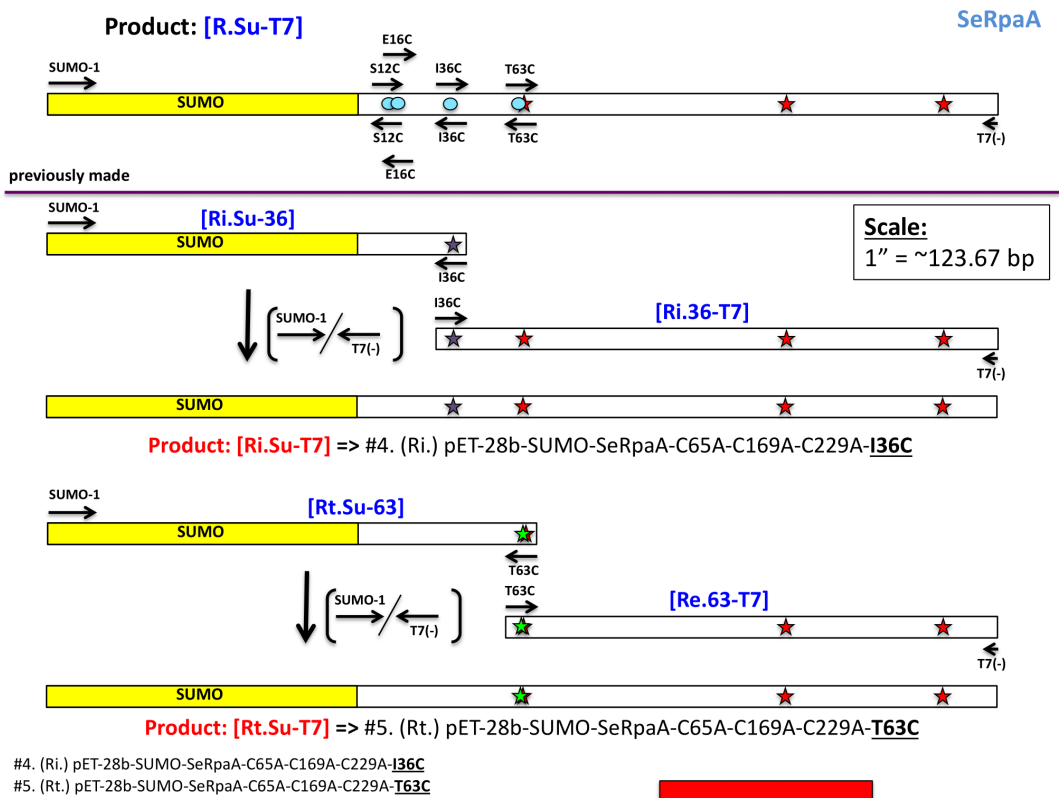
#1. (R.) pET-28b-SUMO-SeRpaA-**C65A**-**C169A**-**C229A**



Product: [Rs.Su-T7] => #2. (Rs.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**S12C**

Product: [Re.Su-T7] => #3. (Re.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**E16C**

#2. (Rs.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**S12C**
#3. (Re.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**E16C**



#4. (Ri.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**I36C**
#5. (Rt.) pET-28b-SUMO-SeRpaA-C65A-C169A-C229A-**T63C**