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Citation	Markson, Joseph Scott and Erin K. O'Shea. The molecular clockwork of a protein-based circadian oscillator. FEBS Letters 583(24): 3938-3947.
Published Version	doi:10.1016/j.febslet.2009.11.021
Accessed	February 19, 2015 10:04:09 AM EST
Citable Link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:8841743
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The Molecular Clockwork of a Protein-based Circadian Oscillator

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Abstract

The circadian clock of the cyanobacterium *Synechococcus elongatus* PCC 7942 is governed by a core oscillator consisting of the proteins KaiA, KaiB, and KaiC. Remarkably, circadian oscillations in the phosphorylation state of KaiC can be reconstituted in a test tube by mixing the three Kai proteins and ATP. The in vitro oscillator provides a well-defined system in which experiments can be combined with mathematical analysis to understand the mechanism of a highly robust biological oscillator. In this Minireview, we summarize the biochemistry of the Kai proteins and examine models that have been proposed to explain how oscillations emerge from the properties of the oscillator's constituents.

Keywords

Biological Oscillator
Circadian Rhythm
Models, Theoretical
Cyanobacteria
Multisite phosphorylation
KaiC

Abbreviations²

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² Abbreviations: *ADP*, adenosine diphosphate; *ATP*, adenosine triphosphate; *P_i*, inorganic phosphate; *U-KaiC*, unphosphorylated KaiC; *S-KaiC*, KaiC phosphorylated only on serine 431; *T-KaiC*, KaiC phosphorylated only on threonine 432; *ST-KaiC*, KaiC phosphorylated on both serine 431 and threonine 432.

Introduction

All organisms have evolved sophisticated mechanisms to sense and respond to changes in their environment. Many environmental perturbations are unpredictable, and cells employ signal transduction mechanisms to sense these perturbations and mount appropriate responses. In contrast, the cycles of light and temperature arising from the rotation of the Earth about its axis – variations that profoundly affect an enormous variety of organisms – are far from random. Rather, the daily rising and setting of the sun is the most universal, ancient, and predictable source of variation in the environment. It comes as no surprise, then, that many organisms from all kingdoms of life have acquired the ability not only to sense, but also to predict, diurnal cycling of the environment. From cyanobacteria to humans, endogenous biochemical oscillators called circadian clocks keep track of the time of day, allowing an organism to coordinate its physiology and behavior with the day/night cycle [1]. These clocks exert their influence over cellular physiology in part by controlling gene expression. In the case of the cyanobacterium *Synechococcus elongatus* PCC 7942, the model system for the cyanobacterial clock, the circadian clock drives rhythmic expression of over thirty percent of the genome [2].

A circadian clock forms an internal representation of external time, but this timepiece continues to run even in the absence of external cues. That is, circadian clocks oscillate with a period of approximately 24 hours (hence their name, meaning “about [*circa*] a day [*dies*]”) under constant environmental conditions (constant light and temperature), a phenomenon known as *free-run* and one of their defining features. Furthermore, the period of oscillation remains around 24 hours across the physiological temperature range despite the high sensitivity of typical biochemical reaction rates to temperature. This *temperature compensation* is a second defining feature of circadian clocks. Finally, a circadian clock would be of little use to an organism if the

clock's phase bore no relationship to the actual time of day. Hence, the phase can be altered by environmental stimuli (e.g., changes in light) in order to synchronize the clock with the diurnal cycle of the environment; moreover, in oscillating environments, the clock adapts to establish a fixed relationship between its phase and that of the environment. These related phenomena, which constitute the third defining feature of a circadian clock, are known as *phase resetting* and *entrainment*, respectively [1].

Generally, circadian clocks are highly robust biological oscillators. For example, the *S. elongatus* clock is resistant to fluctuations produced by metabolic repression [3-4], cell division [5-6], and those arising from the inherent stochasticity of biochemical reactions and gene expression [7-8]. Despite such fluctuations in the cellular milieu, the *S. elongatus* clock oscillates with high precision and minimal damping for weeks in constant conditions [8-9]. Somehow, the clock is sufficiently robust to avoid a loss of synchrony and the resulting spiral into a steady state.

Circadian oscillators evolved long ago, but our understanding of the molecular mechanisms underlying circadian clocks remains murky. These mechanisms have been investigated primarily in cyanobacteria, fungi, flies, plants, and mammals. The prevailing models for the internal timepieces of all but the first of those organisms involve a negative transcriptional feedback loop in which so-called clock genes encode proteins that repress their own transcription. These negative feedback loops typically are intertwined with other feedback loops and are overlaid with posttranslational regulation affecting protein stability, activity, and localization [1]. Disentangling the mechanisms and rigorously testing models of these oscillators have been hampered by the complexity of both the oscillators themselves and the cellular environment in which they are embedded.

An opportunity to break through such complexity recently emerged from investigations of the circadian clock of *S. elongatus*, whose core oscillator consists of just three proteins: KaiA, KaiB, and KaiC. Although the oscillator originally was thought to be a transcriptional feedback oscillator analogous to those found in higher organisms [10], Kondo and colleagues showed in 2005 that the clock of *S. elongatus* requires neither transcription nor translation – oscillations in KaiC phosphorylation state persist in the absence of transcriptional feedback and protein synthesis [4]. Remarkably, the Kai proteins themselves constitute a circadian clock: temperature-compensated circadian oscillations in KaiC phosphorylation can be reconstituted *in vitro* by combining the three Kai proteins and ATP [11]. This three-protein, test-tube oscillator displays all three cardinal properties of a circadian clock: free-run, temperature compensation, and entrainment [11-13]. Oscillations of KaiC phosphorylation free-run for at least ten days *in vitro* [14], and the period of oscillation is temperature-compensated [11,13]. The phase of the *in vitro* clock is phase-shifted by, and entrainable to, temperature shifts [12-13], although it is not entrainable by light, presumably because cellular components required for this property are absent [15-16].

Biochemistry of the Kai oscillator

The ability to reconstitute the oscillator *in vitro* and to mix and match its four components (KaiA, KaiB, KaiC, and ATP) in arbitrary combinations has permitted detailed, quantitative biochemical characterization of the Kai proteins. Structures of all three proteins (or their homologs in related organisms) have been solved by crystallography [17-24] and/or NMR [25], and structural features of various heterocomplexes have been gleaned from NMR [26], electron microscopy [12,20], and small-angle x-ray scattering [27].

KaiC is the only Kai protein with enzymatic activity, and it is the central player in the protein oscillator. An internally duplicated protein, it consists of two homologous domains, the N-terminal CI domain and the C-terminal CII domain, each containing ATPase motifs and belonging to the RecA-DnaB protein family (Figure 1A) [10,21,28-30]. In the presence of ATP, KaiC assembles into a double-doughnut-shaped hexamer, with one lobe consisting of CI domains and the other of CII domains (Figure 1A) [21,31-32]. Within the hexamer, each subunit-subunit interface forms two ATP-binding pockets, one in each lobe; ATP binding promotes KaiC hexamerization by bridging adjacent subunits [21,31]. KaiC possesses autokinase and autophosphatase activities [29,33], phosphorylating and dephosphorylating itself on two adjacent residues – serine 431 (S431) and threonine 432 (T432) – which are buried at the interface between the CII lobes of adjacent subunits [34-35]. The installation of both the kinase and phosphatase activities within the same protein may aid in temperature compensation of the oscillator period by facilitating the coupling of rate changes in one activity to offsetting rate changes in the other.

With two phosphorylation sites, each subunit exists in one of four phosphoforms: unphosphorylated (“U-KaiC”), phosphorylated only on S431 (“S-KaiC”), only on T432 (“T-KaiC”), or on both sites (“ST-KaiC”) [34,36-37]. In the *in vitro* system, the total fraction of KaiC phosphorylated in any form oscillates with a circadian period, with the four phosphoforms appearing in a cyclic order during each period (Figure 1B). A peak of U-KaiC abundance is followed by a peak of T-KaiC and soon after by a peak of ST-KaiC. Finally, a peak of S-KaiC appears before the cycle repeats [36-37]. The phosphoform distribution of KaiC is a major determinant of the phase of the oscillator. Rust et al demonstrated that the initial phase (phosphorylation or dephosphorylation) can be controlled by varying the initial phosphoform

distribution: when KaiC initially is high in S-KaiC and low in T-KaiC, the oscillator starts in the dephosphorylation phase, and vice versa [36].

Studies of the intrinsic autokinase and autophosphatase rates of KaiC and their modulation by KaiA and KaiB have provided insights into the origins of the cyclic pattern of phosphoform abundance. Recent investigations [36-37] of these activities that took into account the different characteristics of the two singly-phosphorylated states have employed two complementary methods. Rust et al [36] fit a kinetic model for phosphoform interconversion to timeseries data from partial reactions (i.e., non-oscillating reactions in which one or more Kai proteins is omitted) to obtain pseudo-first-order rate constants for each phosphorylation and dephosphorylation step. In contrast, Nishiwaki et al [37] studied the behavior of different phosphomimetic KaiCs, that is, mutants designed to act as stable mimics of each of the four possible phosphoforms. The dephosphorylated state of each site was mimicked by mutation to alanine; phosphorylation at S431 was mimicked by mutation to aspartate and at T432 by mutation to glutamate. Each approach has drawbacks. Rate constants obtained from kinetic modeling are model-dependent, and some rate constants are poorly constrained by the data. Moreover, such modeling cannot prove that a particular reaction scheme holds. On the other hand, in some cases, phosphomimetic mutations do not precisely imitate the true (de)phosphorylated forms. In addition, phosphomimetic mutants form hexamers in which all subunits have the same phosphorylation state, a situation that may not occur in the wild-type protein. Nonetheless, the results of these two studies are consistent in many respects.

KaiC is primarily an autophosphatase when incubated alone – at 30° C, the standard temperature for studies of the *S. elongatus* circadian clock, the protein dephosphorylates [4,33-34,36,38], more quickly at T432 than at S431 [36] (Figure 1C). Because S-KaiC is the product

of ST-KaiC dephosphorylation, the abundance of S-KaiC transiently increases before eventually decaying. KaiB has little effect on dephosphorylation at 30° C [33,36,38]. KaiA promotes autophosphorylation of KaiC and inhibits some dephosphorylation steps by binding to the C-terminal tail of KaiC, stabilizing it in a state in which autokinase activity dominates [39] (Figure 1C). As KaiC becomes phosphorylated in the presence of KaiA, U-KaiC is converted to T-KaiC (S-KaiC does not appear to be produced at an appreciable rate from U-KaiC), which in turn is converted to ST-KaiC. ST-KaiC dephosphorylates exclusively at T432, yielding S-KaiC [36-37]. KaiA inhibits the dephosphorylation of S-KaiC [36-37]. Therefore, S-KaiC accumulates slowly in the presence of KaiA. The phosphorylation and dephosphorylation reactions occur on a timescale not much faster than that of the oscillator itself (the rate constants of the kinase and phosphatase reactions are less than $\sim 0.5 \text{ hr}^{-1}$), consistent with the idea that they are rate-limiting steps controlling the dynamics of the oscillator [36].

Rust et al [36] and Nishiwaki et al [37] reached different conclusions about the effects of KaiA on S-KaiC and ST-KaiC. Studies of partial reactions suggest that KaiA keeps the levels of S-KaiC low both by causing rapid rephosphorylation to ST-KaiC and by inhibiting ST-KaiC dephosphorylation [36]. In contrast, Nishiwaki et al showed that while KaiA slows dephosphorylation of T432 in an S-KaiC phosphomimetic mutant (S431D), it causes very little rephosphorylation of T432; formation of S-KaiC from ST-KaiC essentially is irreversible [37]. Hence, the phosphomimetic data suggests that once KaiC becomes doubly phosphorylated, it becomes irreversibly committed to dephosphorylating, while the kinetic studies of the wild-type protein suggest that dephosphorylation is reversible. The details of the interconversion between S-KaiC and ST-KaiC strongly influence the ability of the Rust et al model to generate oscillations [36], and it will be important to resolve these discrepancies.

How does the phosphoform cycle emerge from these properties of the autokinase and autophosphatase activities? Conceptually, a phosphoform cycle appears if the patterns of phosphoform abundance observed in KaiA-KaiC and KaiC-only partial reactions are combined by placing one pattern after the other in time. Therefore, oscillations in phosphoform distribution could result from alternating periods of KaiA activity (phosphorylation phase) and inactivity (dephosphorylation phase) [36]. Indeed, Nishiwaki et al and Rust et al showed experimentally that, during a circadian cycle, the oscillator switches between periods of KaiA activity (in which incorporation of radiolabel from [γ - 32 P] ATP is substantial and in which newly introduced U-KaiC becomes phosphorylated) and inactivity (when radiolabel incorporation is negligible and in which newly introduced U-KaiC remains unphosphorylated) [36-37].

The transition between the phosphorylation and dephosphorylation phase must be mediated by KaiB, as a KaiA-KaiC reaction never enters the dephosphorylation phase [36,38]. Although KaiB does not appear to directly affect the kinetics of phosphoform interconversion at 30° C [33,36], it binds preferentially to certain phosphoforms of KaiC, forming a ternary complex with KaiA that could inactivate KaiA. In an oscillating reaction, the binding of KaiB to KaiC, and hence the co-immunoprecipitation of KaiA with KaiB, closely tracks the abundance of S-KaiC [36-37] (Figure 1D). A complex of KaiB and KaiA binds to phosphomimetic mutants of both S-KaiC and ST-KaiC, albeit more slowly to the latter [37]; whether wild-type ST-KaiC appreciably binds KaiB in the in vitro oscillator remains uncertain. The rhythmic binding of KaiB to KaiC and KaiA could produce fluctuations of KaiA activity, which in turn switch the oscillator between the phosphorylation and dephosphorylation phases. However, other explanations for the trigger of switching between phosphorylation and dephosphorylation have

been proposed. In the section discussing oscillator models later in this review, we will examine these alternative explanations.

In addition to its kinase and phosphatase activities, KaiC has ATPase activity, which hydrolyzes ATP to produce ADP and P_i [40-41], and recent evidence suggests that this enzymatic activity may play an important role in controlling the dynamics of the oscillator. ATPase activity occurs in the CI ATP binding pocket and possibly in the CII pocket [40-41], and it is extraordinarily slow: KaiC turns over an average of 15 molecules of ATP per monomer per day, including ATP used as phosphate donors for autophosphorylation [41]. In an oscillating reaction, the rate of ATP turnover oscillates [41]. Like the kinase and phosphatase rates, the ATP turnover rate (resulting from a combination of the kinase reaction and the hydrolysis of ATP to ADP and P_i) is modulated both by the phosphorylation state of KaiC and by KaiA and KaiB; KaiA enhances the rate of ATP turnover, while KaiB inhibits it [40-41]. The daily ATP consumption is temperature compensated. Interestingly, the ATP turnover rate of KaiC incubated alone also is temperature-compensated, even for phosphomimetics of U-KaiC and ST-KaiC. ATP turnover rates of KaiC period mutants correlate with the period of oscillation *in vivo*, suggesting that like phosphorylation and dephosphorylation, ATPase activity could play a major role in controlling oscillator dynamics [41]. However, the role of ATPase activity in the oscillator remains unclear. Therefore, in this review we focus on the roles of (de)phosphorylation.

Oscillations in phosphorylation state are accompanied by rhythms in the levels of protein complexes formed by KaiA, KaiB, and KaiC [27,38] (Figure 1D). The abundance of KaiB-KaiC and KaiA-KaiB-KaiC complexes fluctuate, with the levels of the KaiB-KaiC complex peaking during the early to mid- dephosphorylation phase and the levels of the ternary complex shortly

thereafter [36-38]. The level of the KaiA-KaiC complex also oscillates with low amplitude [37-38]. While these complexes play key roles in the oscillator by controlling KaiC's enzymatic activities and the activity of KaiA, fluctuations in their levels most likely result from the oscillations in KaiC phosphorylation state, not vice versa. First, the formation of KaiB and KaiA-KaiB complexes with KaiC phosphomimetics indicates a causal role for phosphorylation in complex formation [37]. Second, these complexes seem to be unstable relative to the time scale of oscillations. KaiA and KaiB (un)binding to KaiC can occur on the second timescale, and the reported dissociation constants for KaiA-KaiC and KaiB-KaiC complexes are on the order hundreds of nanomolar to micromolar [27,38]. Interestingly, the oscillator is insensitive to concerted changes in Kai protein concentrations, which can be co-varied across at least an eight-fold range with little effect [36,38]. This robustness is an important systems-level property that mathematical models of oscillator have sought to explain (see section on models below).

KaiC exists primarily as a hexamer throughout the circadian cycle of the *in vitro* oscillator [12,38], but in the preceding discussion of KaiC enzymology, we largely neglected its multimerization. What are the effects of existing as a multimer? The nature and magnitude of hexamer effects remains unknown. In fact, we do not even know the distribution of phosphorylation amongst subunits in a single hexamer. Neglecting hexamer effects is tantamount to assuming that: (a) that hexamerization affects neither the enzymatic properties of its subunits nor their propensity to bind to KaiA and KaiB; (b) hexamer effects exist, but KaiC monomers exchange continuously, rapidly, and randomly amongst hexamers; or (c) all subunits in the hexamer behave in a cooperative, concerted manner, such that each hexamer always consists of identical subunits. The second scenario is ruled out by experimental evidence: Kondo and colleagues have shown that pervasive monomer exchange does not occur. Rather, in an

oscillating reaction, monomers exchange only during the early dephosphorylation phase [14,38]; incubated by itself, unphosphorylated KaiC does not substantially exchange monomers on the hour timescale [38]. The other two scenarios are unlikely. The crystal structure of KaiC shows a hexamer of heterogeneous phosphoform composition (a mixture of ST-KaiC and S-KaiC) [35]. Moreover, features observed in the structure suggest that hexamer composition very likely has an effect on protein function. The phosphorylation sites reside at the interface between subunits, with the phosphates on those sites forming contacts with the neighboring subunit, likely affecting enzymatic activity, nucleotide binding, and hexamer stability [21,35]. In addition, the C-terminal tails of the subunits, the conformations of which govern KaiC kinase activity, form a hydrogen-bonding network with one another, suggesting a coupling of their conformations [21,39].

Modeling a biological oscillator: General considerations

How do oscillations in KaiC phosphorylation state emerge from the biochemical properties of the three Kai proteins combined with ATP? Oscillations are a system-level property: all three proteins and ATP are required for oscillations in KaiC phosphorylation [38]. Oscillations are sufficiently complex phenomena that intuition alone is inadequate for understanding them [42]. To elucidate the origins of oscillation, we must turn to mathematical modeling. An iterative process of constructing models, using them to make experimentally-testable predictions, performing the experiments, and revising the models based on the experimental results is required for assessing and improving our understanding of the oscillator.

Circadian clocks are limit-cycle oscillators that follow a single, closed-loop trajectory (the limit cycle) through phase space (the multi-dimensional space encompassing all possible

states of the system) under a given set of conditions [1]. Perturbations away from the limit cycle are followed by a return to the same trajectory, making the oscillator resistant to fluctuations in conditions. The system spontaneously approaches the limit cycle from a range of initial conditions, getting progressively closer to the cycle as time goes on (hence the name of *limit* cycle). The mechanisms generating the oscillations must actively keep the system from spiraling into a stable steady state in which all dynamical variables remain constant in time; instead, oscillation-generating mechanisms push the system toward the limit cycle and far away from equilibrium [42].

What features must the Kai system possess in order to generate oscillations? Considering the alternating phases of phosphorylation and dephosphorylation of KaiC, one requirement for limit-cycle oscillation becomes immediately apparent: there must be a time delay between the beginning of the phosphorylation phase and the switch to the dephosphorylation phase, and vice versa [43]. This temporal separation is necessary to keep the system away from a stable steady state in which the concentrations of all phosphoforms are constant. If phosphorylation were immediately followed by a tendency to dephosphorylate, the two reactions would be exactly balanced; although ATP consumption would continue, the system would end up in a stable steady state and no oscillations would occur. Time delays are required in order to cause the system to persistently overshoot and undershoot the steady state, keeping it away from the steady state [44-45]. Time delays also provide a mechanism for maintaining directionality – phosphorylating at one time and dephosphorylating at another. (In mechanical systems, inertia can maintain directionality, but chemical systems lack inertia.)

However, time delays are not sufficient for oscillations to occur [46]. Consider the hypothetical scenario shown in Figure 2A, in which KaiC is forced to cycle through the four

phosphoforms (independently of KaiA or KaiB) via the intermediate, singly phosphorylated states that provide time delays. Because individual KaiC molecules are forced to cycle, it may appear as though this scheme should generate population-level oscillations in the phosphoform distribution of the ensemble of KaiC molecules. But oscillations cannot occur – the system always spirals into a stable fixed point (Figure 2A). The reason is that each step in the phosphorylation cycle of an individual KaiC molecule is a stochastic chemical reaction; the time it takes for each molecule to undergo a phosphorylation or dephosphorylation event varies due to thermal fluctuations. These stochastic fluctuations cause some KaiC molecules to traverse the cycle faster than others, resulting in desynchronization. The phosphoform distribution of the ensemble of KaiC molecules approaches a steady-state value as individual molecules become increasingly desynchronized.

The failure of this scheme (Figure 2A) to generate oscillations illustrates the second major requirement for oscillations – a mechanism for maintaining sufficient synchrony amongst KaiC molecules to sustain population-level oscillations. Feedback can generate this synchrony. KaiC molecules that progress too quickly through the cycle must act (directly or indirectly) to speed up the laggards until they catch up, or hold them back until the leaders can return to the starting point; alternatively, the laggards could speed up or slow down the leaders. Feedback causes an oscillator to switch (rapidly in some systems and more slowly in others) back and forth between two opposing states – in our case, phosphorylation and dephosphorylation. At the transition between those two states (which can be gradual, rather than instantaneous), some molecular event(s) cause most if not all components to switch states, thereby synchronizing them. Synchrony amongst KaiC hexamers also could be promoted by monomer shuffling. Finally, limit cycle oscillations require nonlinearities [47], and in a chemical system require an

energy source. Cooperativity, feedback, and time delay schemes can provide nonlinearities, and ATP consumption provides energy to the Kai oscillator.

Models of the Kai oscillator

Many groups have proposed quantitative models of the oscillator, typically expressed in differential equations describing the rates at which key chemical species (e.g., forms of KaiC and/or Kai protein complexes) are produced and destroyed [12,27,43,46,48-54]. Here we examine these models, focusing on the sources of time delays and synchrony in each.

Time delays/temporal separation of phases

Prior to the discovery of the phosphoform cycle in 2007 [36-37], a major challenge for modelers was to explain the temporal separation between dephosphorylation and phosphorylation. If one considers only the total phosphorylation level of KaiC (i.e., either the proportion of KaiC phosphorylated anywhere or the number of phosphates per KaiC unit), then phosphorylating and dephosphorylating KaiC molecules pass through the same phosphorylation states. Without a biochemical distinction between the two phases, it becomes more difficult (but not impossible; see, for example, ref. [52] or the Supporting Information in ref. [54]) to explain their temporal separation and the maintenance of reaction directionality. Models that neglect the phosphoform cycle typically achieve temporal separation by assuming that phosphorylating and dephosphorylating KaiC differ somehow in conformation and/or in interaction with KaiA/KaiB, and often need to assume that these differences are long-lived [27,46,48-50,53-55]. However, experimental evidence for conformational states or protein complexes with such long lifetimes (~12 hours) is lacking. In fact, KaiA and KaiB binding and unbinding to KaiC can be rapid [38],

and KaiC passing through the middle of the dephosphorylation phase can be rapidly switched to a phosphorylating state when a high concentration of KaiA is added to the reaction [36].

Similarly, following the removal of KaiA from a KaiA-KaiC reaction, dephosphorylation rapidly commences [36,38]. Therefore, while KaiC likely has multiple conformational states, each with different kinase, phosphatase, and ATPase rates, these states are short-lived and rapidly interconvertible.

The need to posit kinetically-stable, non-covalent states to explain temporal separation was obviated by the discovery that the phosphoform population during phosphorylation and dephosphorylation is quite different: high levels of T-KaiC and low levels of S-KaiC mark the phosphorylation phase, and vice-versa during dephosphorylation, even though the total phosphorylation levels may be the same [36-37]. The intermediate phosphorylation states during the phosphorylation and dephosphorylation phases provide a source of time delays [43,45]. Moreover, the different phosphoform distributions during the two phases establish a biochemical distinction between them, enabling the oscillator to “know” in which direction it should be going. Critical to establishing those different phosphoform distributions, and the phosphoform cycle more generally, are the intrinsic directionality of KaiC phosphorylation and dephosphorylation, with T432 always phosphorylated and dephosphorylated before S431. Molecular mechanisms for the directionality of KaiC (de)phosphorylation have been proposed based on the KaiC crystal structure [56]; this proposal needs to be tested by obtaining and comparing structures of KaiC in different phosphorylation states.

Synchronization

The mechanism by which synchronization is achieved remains under debate. Two general synchronization mechanisms, not mutually exclusive, have been proposed (Figure 3). In one mechanism (Figure 3A), synchronization is achieved primarily by modulation of KaiA and/or KaiB activity by specific states of KaiC [43,49,52,54-55]. In most models invoking this mechanism, stoichiometric control of KaiA activity by differential sequestration on or inhibition by KaiC (directly or via KaiB) is central. In these models, the concentration of KaiA is limiting for phosphorylation; when specific states of KaiC inhibit the activity of KaiA, all KaiC in the reaction – regardless of its state – is affected, since KaiA controls the balance between the kinase and phosphatase activities of all KaiC molecules. In the second synchronization mechanism (Figure 3B), synchrony is achieved through direct interhexamer communication via either monomer exchange or autocatalysis [12,46,48,50,53]. KaiA and KaiB cause KaiC molecules to transition between different states, but they are not the proximal source of feedback and synchronization. Note that the two mechanisms are not mutually exclusive, and in some models both mechanisms operate to a greater or lesser extent.

To illustrate the first category of synchronization mechanisms, we examine the experimentally-constrained model proposed by Rust et al [36] (Figure 2B and Figure 3A). In this model, synchronization occurs via inactivation of KaiA by KaiB in the presence of the last phosphoform to accumulate during the phosphorylation phase (S-KaiC), causing autocatalytic production of S-KaiC (positive feedback) and global inhibition of phosphorylation of other species (negative feedback). This inhibition of KaiA is supported by the observations that (a) KaiB's binding to both KaiC and KaiA closely tracks the abundance of S-KaiC, and (b) KaiA activity is absent during the dephosphorylation phase, the time at which S-KaiC abundance is high. Uniquely amongst published models, the Rust et al model was constructed with

experimentally-measured phosphorylation and dephosphorylation kinetics for each phosphoform interconversion. Using these measured kinetics and assuming stoichiometric inhibition of KaiA by S-KaiC (via KaiB), the model generates limit-cycle oscillations with the correct period and pattern of phosphoform abundances (Figure 2B) [36]. The model does not invoke any role for direct interhexamer communication in synchronization. Hence, stoichiometric inhibition of KaiA by a form of KaiC is sufficient for maintaining adequate synchrony. Clodong and colleagues similarly concluded that direct interhexamer communication is not required in a model in which dephosphorylating KaiC stoichiometrically inhibits KaiA and is irreversibly committed to dephosphorylating [49].

In contrast, in models invoking the second synchronization mechanism, KaiC hexamers directly communicate with one another to synchronize their phases. In one model [50], a complex of KaiA and phosphorylated KaiC acts autocatalytically, accelerating the association of KaiA with KaiC and phosphorylation of the latter; no experimental evidence for such autocatalysis has been reported. Other models [12,46,48,53] employing the second synchronization mechanism rely on monomer exchange/shuffling between KaiC hexamers to maintain synchrony. Monomer exchange has the obvious effect of averaging hexamer compositions, thereby reducing inter-hexamer variability arising from the inherent stochasticity of kinase and phosphatase reactions. Indeed, such shuffling occurs during the early dephosphorylation phase. However, some models [46,53] assume that monomer exchange has a much stronger effect on the oscillator than mere averaging. In these models, highly phosphorylated hexamers that have just entered a dephosphorylating state not only exchange monomers amongst themselves, but also induce exchange with hexamers in the normally non-exchanging, phosphorylation-prone state (Figure 3B). This results in hybrid hexamers

containing a mixture of subunits in phosphorylation- and dephosphorylation-prone states. The dephosphorylation-prone subunits act dominantly to switch the phosphorylation-prone subunits into the dephosphorylating state. This process results in the synchronization of KaiC hexamers into the dephosphorylation-prone state (Figure 3B).

Ito and colleagues experimentally probed the mechanism of synchronization by mixing oscillators in different phases, then monitoring the total phosphorylation level of KaiC in the mixtures [14]. All mixtures continued oscillating, but dephosphorylating reactions dominated in determining the resulting phase: when a dephosphorylating reaction was mixed with a phosphorylating reaction, the phase of the mixture was similar to that expected if the dephosphorylating reaction had been incubated alone. The authors explain the dominance of dephosphorylating over phosphorylating reactions in terms of induced monomer exchange, with a secondary role for phase-dependent sequestration of KaiA (Figure 3B). They show that six hours after mixing a phosphorylating and dephosphorylating reaction, more exchange has occurred between KaiC molecules originating from the two original hexamer pools than would be expected if those pools were unaffected by one another. Ito et al interpret this observation in terms of a process in which the dephosphorylating hexamers induce monomer exchange with the otherwise non-exchanging, phosphorylating hexamers, resulting in synchronization into a dephosphorylating state, as described above. This process is reinforced by the inactivation of KaiA by KaiB and KaiC. The switch back to the phosphorylation phase is caused by the release of KaiA inhibition [14].

However, these results also could be explained in terms of the KaiA-inhibition mechanism of synchronization. Although the authors [14] did not monitor the phosphoforms distribution of KaiC, we know that dephosphorylating and phosphorylating oscillators contain

very different distributions. KaiC in dephosphorylating oscillators is enriched in S-KaiC, which (via KaiB) could inactivate KaiA from the phosphorylating reaction following mixing [36-37]. KaiA activity then would drop, in turn causing dephosphorylation of the KaiC originating from the phosphorylating mixture. Hence, KaiC from both origins would dephosphorylate – and because dephosphorylating hexamers mix with each other [14,38], the two pools of hexamers exchange monomers. A KaiA-inhibition mechanism also is consistent with the observation that the dominance of the dephosphorylating reaction requires that it constitute over 10% of the final mixture [14]; this concentration threshold is easier to explain with stoichiometric inhibition of KaiA than with induced monomer exchange. In light of these considerations, the extent to which directly-induced monomer exchange plays a role in the *in vitro* oscillator remains unclear.

Open questions and future directions for the *in vitro* oscillator

Below, we highlight some of the fundamental unresolved questions about the Kai oscillator, the answers to which will be required to build a coherent understanding of how robust oscillations emerge from the biochemical properties of the Kai proteins.

- ***Hexamer effects:*** What phosphorylation states does a hexamer visit? That is, what combinations of subunit phosphorylation states appear in hexamers? How much variability exists amongst hexamers at any given time? What are the effects of subunit composition on the enzymatic and protein binding activities of the hexamer? How cooperative are subunit phosphorylation and dephosphorylation in the hexamer?

- ***Role of ATPase activity:*** What is the role of ATPase activity [40-41] (as distinct from kinase activity)? Does ATP hydrolysis provide energy with which to power a conversion of KaiC into a high-energy state?
- ***The pacesetter:*** What is the fundamental timekeeper in the Kai oscillator? Is it ATPase activity, kinase and phosphatase activity, or some combination of the two? Perhaps all three reactions are controlled by a more fundamental process, such as the conversion of KaiC into a rare conformational state in which those reactions actually occur. Most likely, the rate of conformational change would be influenced by the phosphorylation state of KaiC and possibly the other products of the enzymatic activities (ADP and P_i).
- ***Synchronization:*** What maintains sufficient synchrony in the oscillator to avoid dephasing due to the inherent stochasticity of chemical reactions? Is it rhythmic inhibition of KaiA and/or monomer exchange? If induced monomer exchange occurs, what is the molecular mechanism by which some special state(s) of KaiC induce exchange with otherwise non-exchanging KaiC?
- ***The structural biology of oscillator dynamics:*** While atomic structures of each of the Kai proteins have been solved, the structural bases for the many unique and complex behaviors of the oscillator remain unclear. For example, what is the structural basis for the ordered (de)phosphorylation executed by KaiC, and why is the rate of KaiC ATPase activity independent of temperature? Further experimental and computational studies on the structural dynamics of the Kai proteins should provide insights into these exceptional features of the Kai oscillator. Obtaining structures of KaiC in different phosphorylation states and in

complex with KaiA and/or KaiB will be of critical importance to those investigations.

In studying the protein oscillator, we must keep in mind the cellular context in which it functions *in vivo*. In the cell, the protein oscillator is embedded within a transcriptional feedback loop and interacts with components of the input and output pathways [5,53]. The cellular environment fluctuates, and the oscillator has evolved to function robustly in the face of such noise. How do the properties of the protein oscillator relate to these evolutionary constraints, and what molecular mechanisms give rise to those properties?

- ***Temperature compensation:*** What makes the oscillator's period essentially insensitive to temperature? In principle, a temperature-independent period could be achieved by a balance between period-lengthening and period-shortening processes that are themselves sensitive to temperature, and/or with period-determining processes that are *insensitive* to temperature. Like the ATPase activity of KaiC [41], the activity of a key kinase in the mammalian clock appears to be minimally sensitive to temperature [57]. Early work [4] suggested that the kinase and phosphatase activities of KaiC also may be minimally sensitive to temperature, although individual rate constants were not measured. However, not all of the fundamental processes governing the Kai oscillator can be insensitive to temperature; if they were, the experimentally-observed resetting by temperature shifts [13] could not occur. The paradox between a temperature-insensitive period and a temperature-responsive phase in the Kai oscillator remains unresolved.

- ***Extent of robustness:*** How sensitive is the oscillator to the stoichiometry of the Kai proteins? More generally, how robust is the oscillator to *fluctuations* (in contrast to sustained changes) in protein concentrations, which inevitably occur in vivo?
- ***Mechanism of phase resetting:*** While the *in vitro* Kai oscillator is not affected by fluctuations in light, the *in vivo* clock does reset in response to pulses of darkness. What is the mechanism by which dark pulses are sensed and the Kai oscillator reset? The protein CikA is involved in dark pulse-induced phase resetting [16], but the way in which it impinges on the Kai oscillator is unknown.
- ***Role of transcription/translation feedback in vivo.*** In vivo, the Kai protein oscillator feeds back onto itself by rhythmically modulating the production rate of new KaiB and KaiC molecules, causing the concentrations of these species to oscillate. Interestingly, newly transcribed/translated (and hence unphosphorylated) KaiC appears to be produced maximally when existing KaiC is highly phosphorylated. What are the consequences of adding U-KaiC into the system at this time? More generally, what are the effects of the transcriptional feedback on the protein oscillator? Does it enhance robustness of the oscillator to intracellular and extracellular (environmental) perturbations/noise?

A recent study [58] showed that, in vivo, certain non-phosphorylatable phosphomimetic mutants of KaiC designed to mimic either U-KaiC or ST-KaiC can generate oscillations in a luciferase-based gene expression reporter in the absence of wild-type KaiC. However, the amplitude of these oscillations was reduced relative to that of the wild-type oscillator. These data indicate that

oscillatory phosphorylation is not the sole source of rhythmicity in vivo. Perhaps the combination of a phosphorylation-independent activity of KaiC (e.g., ATPase activity) and transcriptional feedback generates the weak rhythms. The authors [13] suggest that such phosphorylation-independent oscillations couple cooperatively with the oscillations in phosphorylation state to generate the exceptional robustness of the clock in vivo.

In answering these questions, the construction and interrogation of quantitative models of the Kai oscillator will be essential. These models need to be evaluated by subjecting non-trivial predictions to experimental tests. One of the simplest ways to test a model is to examine its ability to predict the behavior of Kai reactions of different stoichiometries. The oscillator's behavior as the concentrations of KaiA or KaiB are lowered across the failure threshold (bifurcation point) has been examined, as have the effect of concerted changes in the concentrations of all three proteins [38], but a systematic exploration of oscillator dynamics as a function of stoichiometry has not been performed. The effects of stoichiometry are easily explored experimentally, and generally different models will make different predictions about the boundaries of oscillation and the sensitivity of period, amplitude, and waveform to stoichiometry. Through an iterative process of experiment and modeling, a fuller, more holistic understanding of the Kai protein oscillator will be obtained. Ultimately, we may find that the mechanisms that make the Kai oscillator an extremely robust timepiece are employed by other biological oscillators, including the eukaryotic circadian clocks that *prima facie* appear so different from the cyanobacterial clock.

Acknowledgements

We thank Michael Rust for many insightful comments and stimulating discussions about the Kai oscillator, and M.R. and Vikram Vijayan for comments on the manuscript.

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Figure Legends

Figure 1. Biochemical features of the Kai oscillator.

(A) The structure of KaiC (PDB 2GBL). (*Upper left*) The surface of a KaiC hexamer viewed from the side of the double-doughnut-shaped multimer. Each subunit is shown in a different color. ATP molecules bound at the interface between adjacent subunits are shown in yellow; only the edge of the adenine base is visible, with the remainder of the nucleotide buried in the subunit-subunit interface. (*Lower left*) The top of the KaiC hexamer, viewed from the CII side, with the C-terminal tails (residues beyond 497) omitted for clarity. This view is obtained by rotating *upper left* view by 90° out of the plane of the page. (*Right*) Cutaway view showing the locations of the ATPs and the two phosphorylation sites at the subunit-subunit interface. Chain E has been removed to reveal the buried ATP and phosphorylation sites. The ATP molecules, phosphorylated S431 (chain F), and phosphorylated T432 (chain F) are highlighted. Figures were rendered using VMD [59].

(B) Rhythmic phosphorylation of KaiC in a KaiA-KaiB-KaiC reaction. (*Top*) Image of an SDS-PAGE gel used to resolve the four phosphoforms of KaiC. The time in hours at which each sample was acquired is indicated; the three proteins were mixed and placed at 30 °C at time zero. (*Bottom*) Quantification of the KaiC phosphoform distribution as a function of time. U-KaiC, unphosphorylated KaiC; T-KaiC, KaiC phosphorylated only on T432; S-KaiC, KaiC phosphorylated only on S431; ST-KaiC, KaiC phosphorylated on both S431 and T432; Total, percentage of KaiC phosphorylated at either or both residues (S-KaiC + T-KaiC + ST-KaiC).

(C) Diagram showing the major KaiC autokinase and autophosphatase reactions that occur in the presence or absence of KaiA, according to Rust et al [36] and Nishiwaki et al [37]. In the fitted rate constants obtained by Rust et al [36], the rate constant for the dephosphorylation of ST-KaiC to T-KaiC was nonzero, but we now believe that this is a fitting artifact and that the rate constant is, in fact, negligible, consistent with the phosphomimetic data of Nishiwaki et al; therefore, we do not show an arrow from ST-KaiC to T-KaiC. The arrow denoting the phosphorylation of S-KaiC to produce ST-KaiC is colored gray to indicate the disagreement between the results of Rust et al [36] (which suggest that the process occurs at an appreciable rate) and those of Nishiwaki et al [37] (which suggest that the process does not occur). As the rate at which S-KaiC can be phosphorylated to yield ST-KaiC in the presence of KaiA plays an important role in the model of Rust et al, it is important to further investigate whether this process occurs.

(D) A schematic showing key Kai protein complexes that appear during a circadian cycle. KaiA symbols represent dimers, while KaiB symbols represent either dimers or tetramers. At any point in time, the composition of Kai protein complexes is heterogeneous, and a large fraction of KaiC is not bound by KaiA or KaiB; here, only a single complex is shown at each time point to emphasize when key complexes appear. The precise stoichiometries of the various Kai protein complexes remain uncertain, and we have selected arbitrarily a few possible stoichiometries. Also, while phosphorylated S431 (pS431) and phosphorylated T432 (pT432) are located at the interface between subunits (see Figure 1A), here we show them on the outer surface of the subunits for clarity. Active KaiA shuttles amongst KaiC hexamers in the phosphorylation phase, promoting ordered KaiC phosphorylation. KaiB binds primarily to S-KaiC (which appears through dephosphorylation of ST-KaiC), but also may bind to ST-KaiC. Bound KaiB inactivates KaiA, possibly through the formation of KaiA-KaiB-KaiC ternary complexes, which accumulate

as S-KaiC levels rise during the mid-dephosphorylation phase through decay of the ST-KaiC reservoir. As levels of S-KaiC themselves decay through dephosphorylation, KaiB binding – and hence KaiA inhibition – is relieved, releasing active KaiA to begin the phosphorylation cycle anew.

Figure 2. Generation of oscillation by KaiA, KaiB, and KaiC.

(A) *Top*, A hypothetical cyclic reaction scheme for KaiA- and KaiB-independent KaiC phosphorylation and dephosphorylation that does not generate oscillations. *Middle*, Timecourse of phosphoform abundance with all phosphoform interconversions modeled as first-order reactions with rate constants of 0.116 h^{-1} . *Bottom*, Trajectories of the system starting with various initial conditions; black arrows denote directionality of the trajectories. Notice that all trajectories spiral into a stable fixed point, marked with a solid black circle.

(B) *Top*, Diagram of the model proposed by Rust et al [36] for limit cycle oscillations of the Kai oscillator. Lines emanating from KaiA ending in an arrowhead (black) or bar (gray) indicate stimulation or repression, respectively, of the transition toward the indicated form of KaiC; only the dominant effects of KaiA are shown. S-KaiC inactivates KaiA via KaiB. Figure is from Rust et al, *Science* **318**: 809-812 (2007). Reprinted with permission from AAAS. *Middle*, Timecourse of phosphoform abundance modeled using the same rate constants employed in Figure 4B of Rust et al [36]. *Bottom*, Trajectories of the system starting with various initial conditions; black arrows denote directionality of the trajectories. Notice that all trajectories, regardless of initial conditions, approach the limit cycle, which is indicated by red arrows.

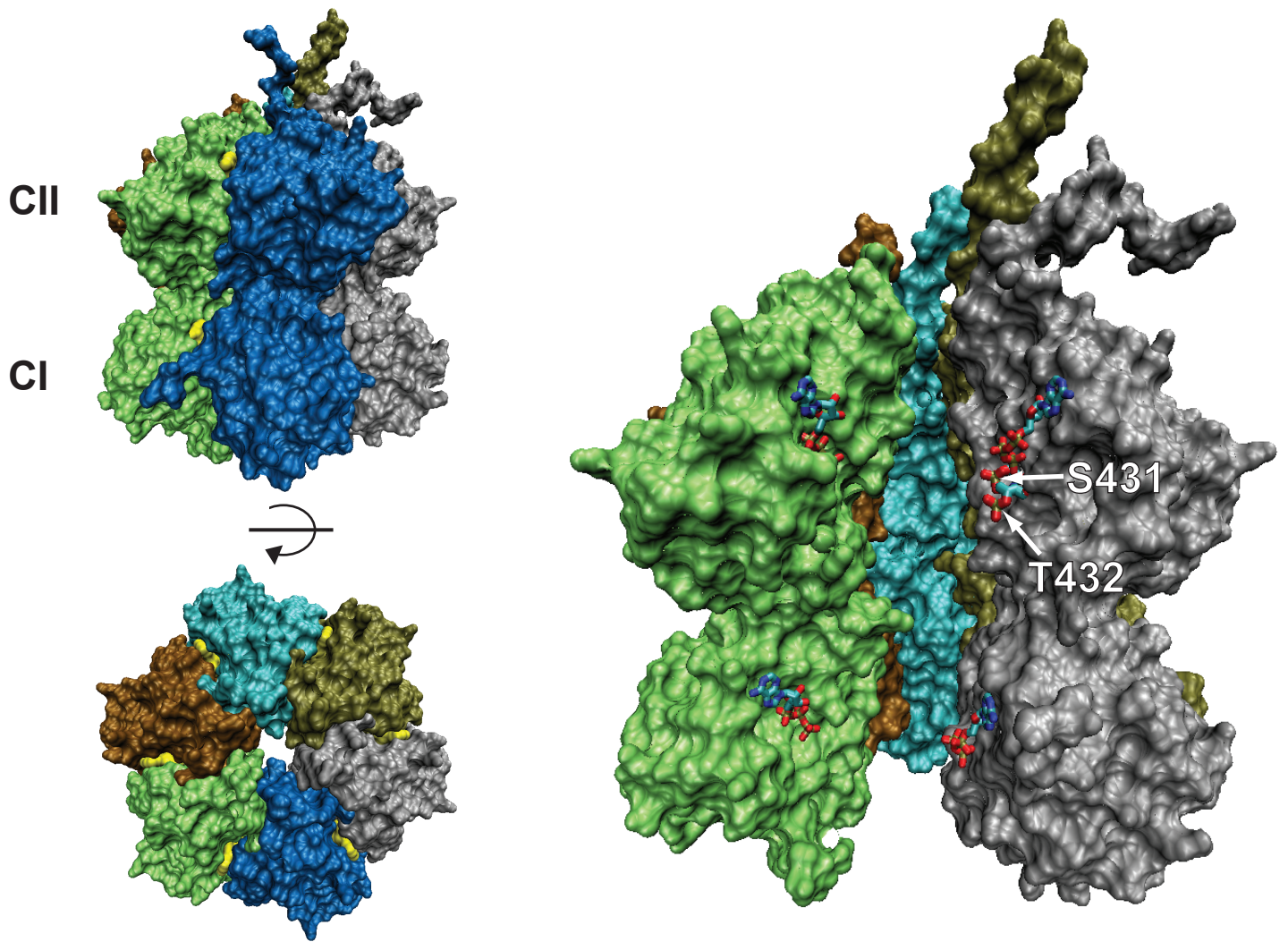
Figure 3. Two KaiC synchronization mechanisms.

(A) Synchronization through stoichiometric control of KaiA activity by differential inhibition by KaiB and KaiC. Shown is a schematic demonstrating how the global inhibition of KaiA activity by S-KaiC (via KaiB) prevents the phosphorylation of all KaiC hexamers in the reaction, according to the model of Rust et al [36]. Any hexamers with a phosphoform composition typical of the phosphorylation phase begin to dephosphorylate as a result of the loss of KaiA activity.

(B) Synchronization through direct interhexamer communication via monomer exchange/shuffling. Shown is the scheme described by Ito et al [14] in which synchronization is effected through forced monomer exchange between phosphorylating KaiC and a special, dominant form of dephosphorylating KaiC that appears in the early dephosphorylation phase (grey box). Inhibition of KaiA by dephosphorylating KaiC (via KaiB), and the release of that inhibition following dephosphorylation, also plays a role in maintaining synchrony. Adapted from ref. [14] by permission from Macmillan Publishers Ltd: Nature Structural and Molecular Biology, copyright 2007.

Figure 1

A



B

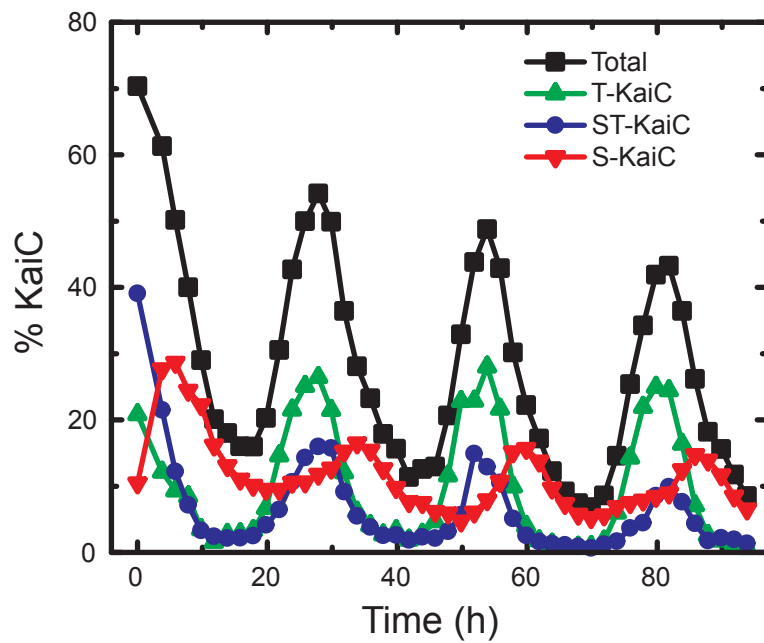
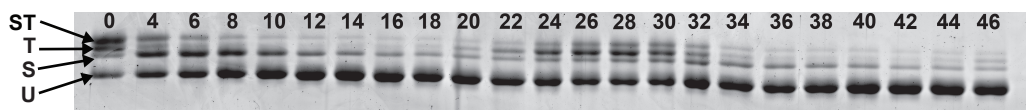
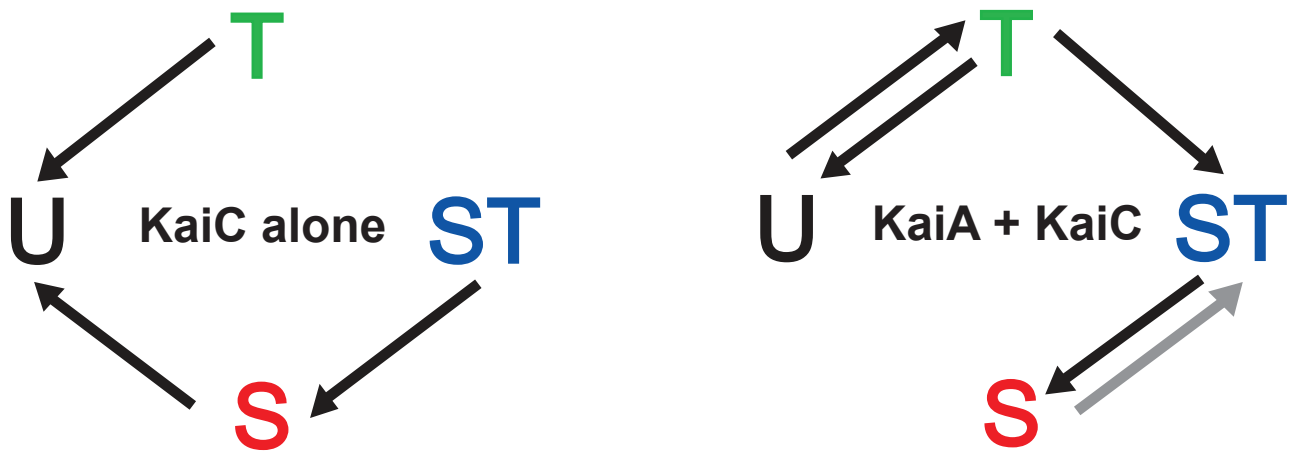


Figure 1

C



D

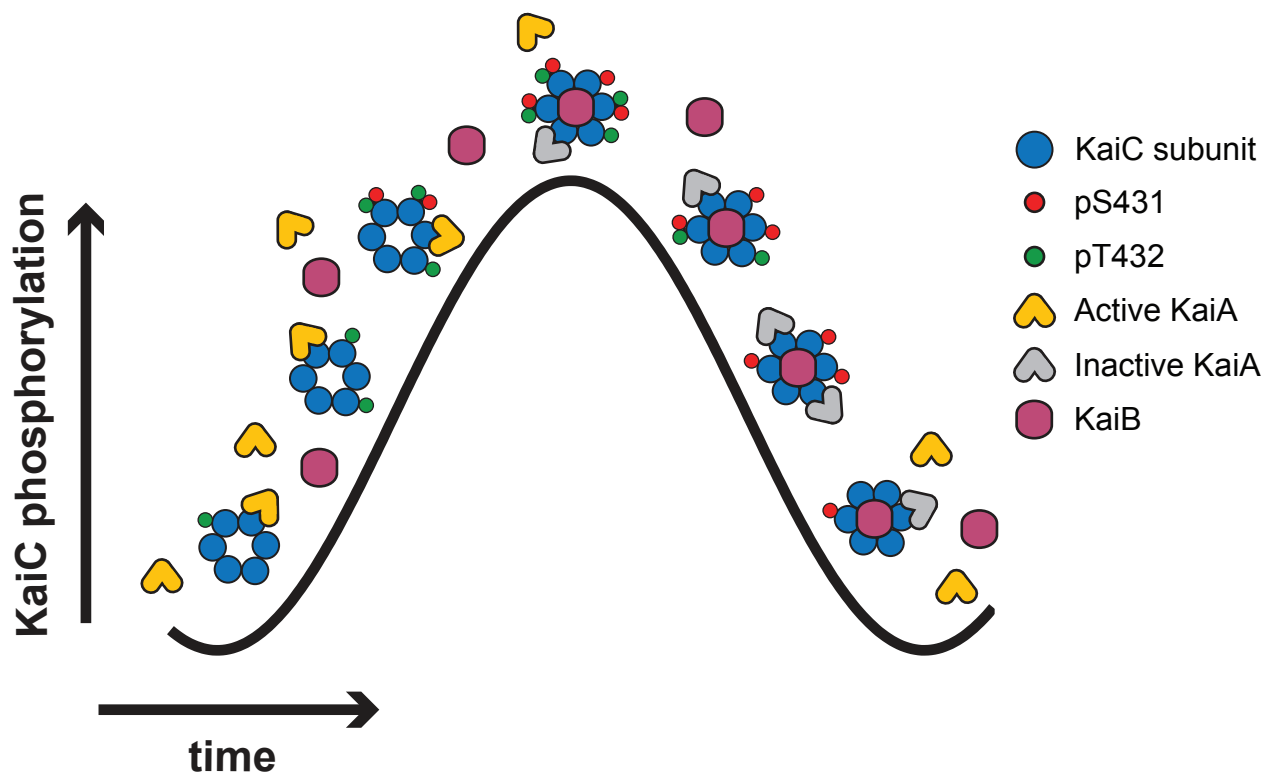
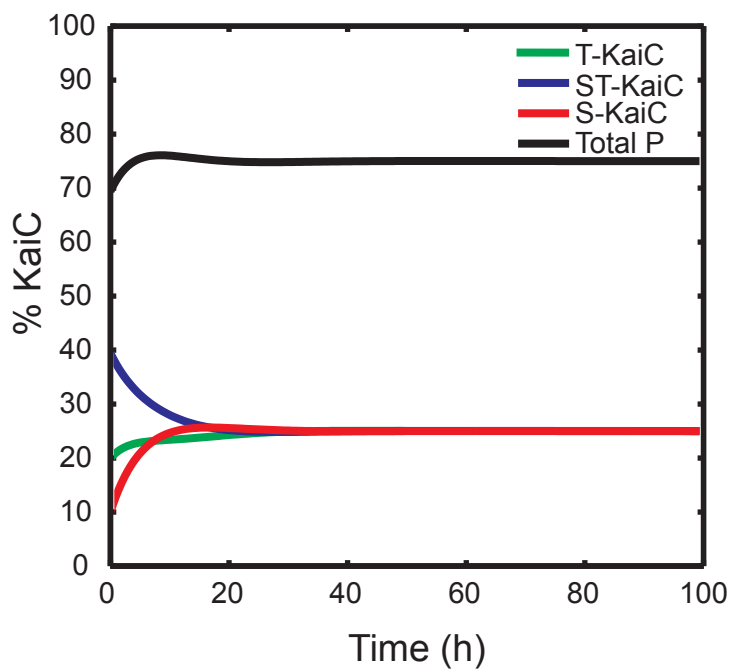
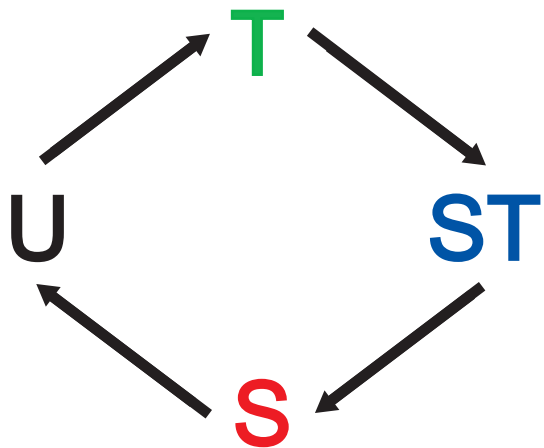


Figure 2

A



B

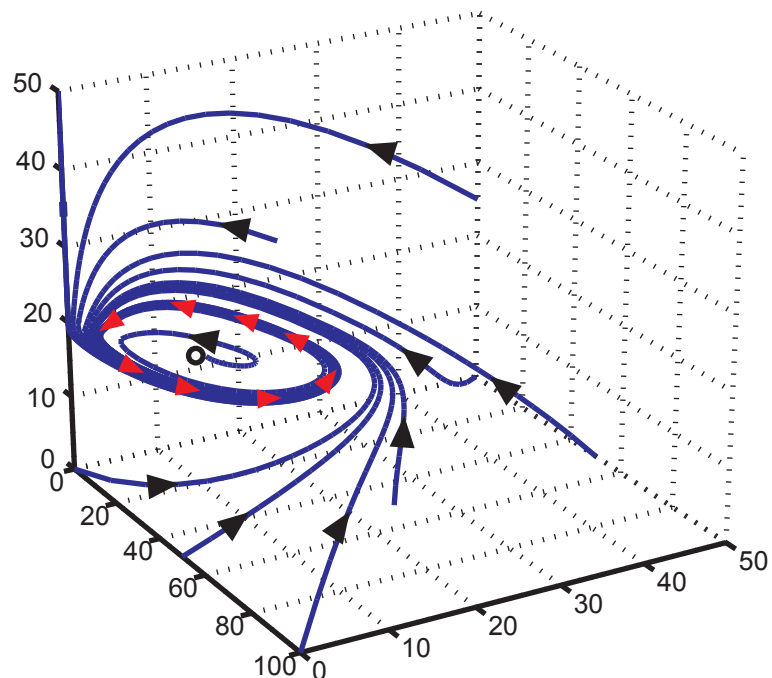
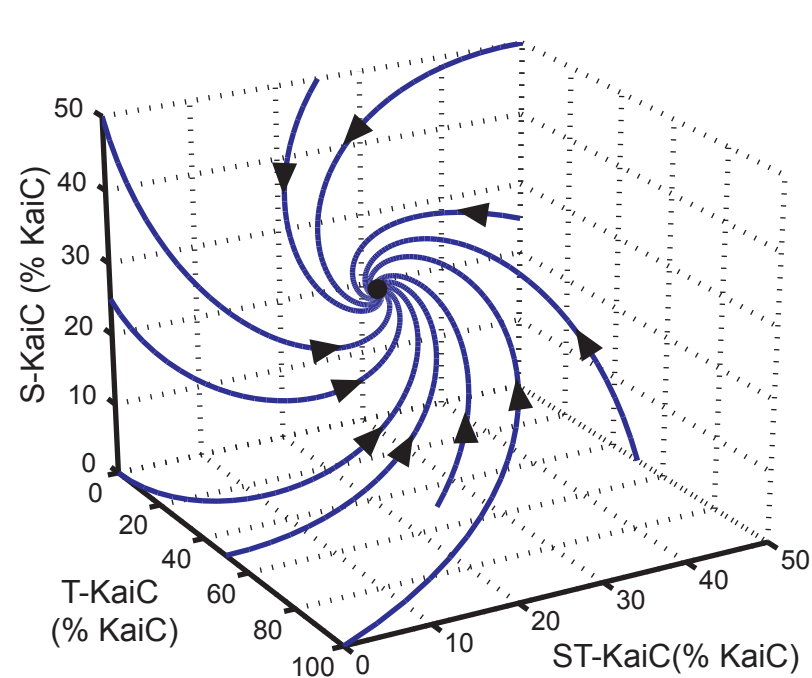
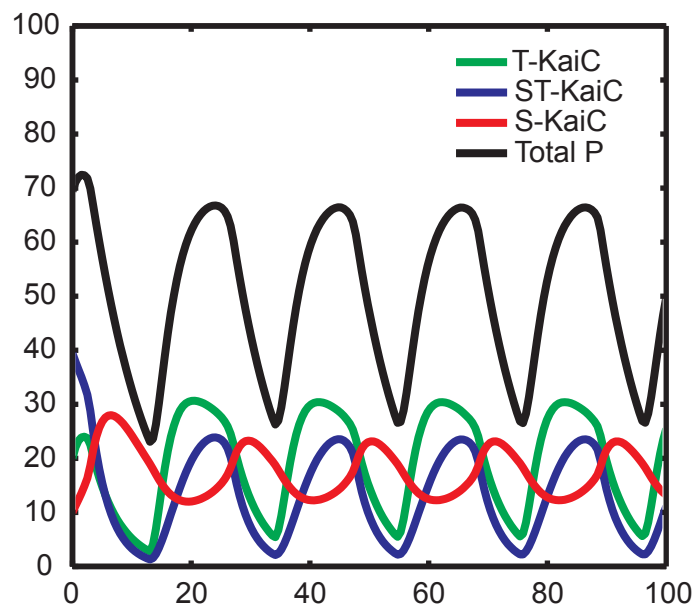
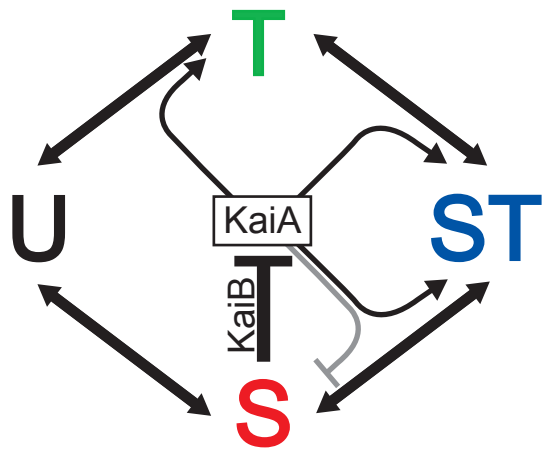
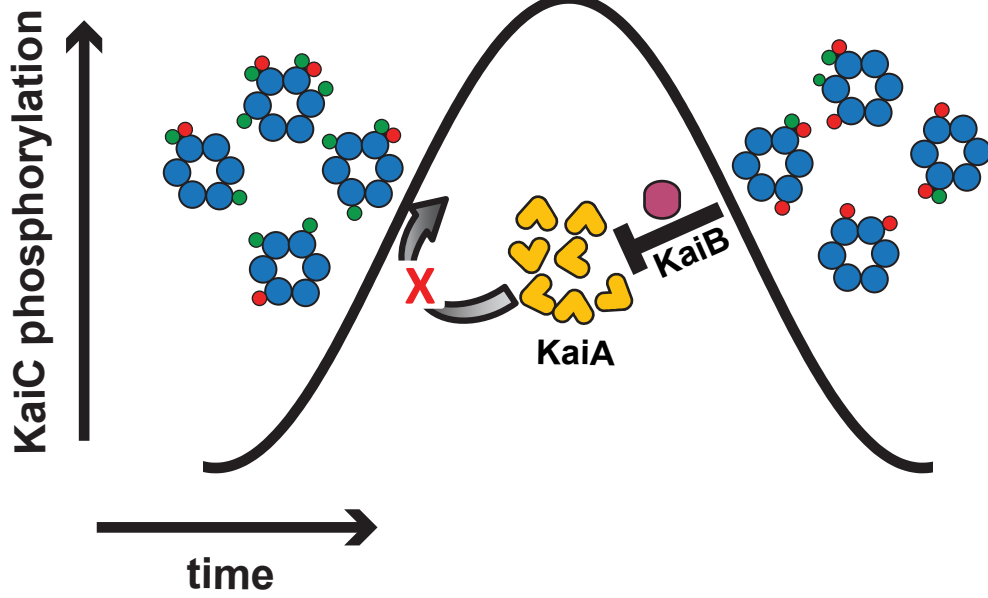


Figure 3

A



B

