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Structural Insights into a Circadian Oscillator

Carl Hirschie Johnson^{1*}, Martin Egli², and Phoebe L. Stewart³

¹*Dept. of Biological Sciences, Vanderbilt University, Nashville, USA*

²*Dept. of Biochemistry, Vanderbilt University, Nashville, USA*

³*Dept. of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, USA*

Abstract

An endogenous circadian system in cyanobacteria exerts pervasive control over cellular processes including global gene expression. Indeed, the entire chromosome undergoes daily cycles of topological changes and compaction. The biochemical machinery underlying a circadian oscillator can be reconstituted in vitro with just three cyanobacterial proteins, KaiA, KaiB, and KaiC. These proteins interact to promote conformational changes and phosphorylation events that determine the phase of the in vitro oscillation. The high-resolution structures of these proteins suggest a ratcheting mechanism by which the KaiABC oscillator ticks unidirectionally. This post-translational oscillator may interact with transcriptional and translational feedback loops to generate the emergent circadian behavior in vivo. The conjunction of structural, biophysical, and biochemical approaches to this system reveals molecular mechanisms of biological timekeeping.

Many biological processes undergo daily (circadian) rhythms that are dictated by self-sustained biochemical oscillators. These circadian clock systems generate a precise ~24 h period in constant conditions (constant light and temperature) that is nearly invariant at different temperatures (temperature compensation)⁽¹⁾. Circadian clocks also show entrainment to day and night, predominantly mediated by the daily light/dark cycle, so that the endogenous biological clock is phased appropriately to the environmental cycle (2). These properties—especially the period's long time constant and temperature compensation—are difficult to explain biochemically. Full understanding of these unusual oscillators will require knowledge of the structures, functions, and interactions of their molecular components.

Pervasive circadian rhythms in a bacterium

We study the components of the biological clock in the prokaryotic cyanobacterium, *Synechococcus elongatus*, which programs many processes to conform optimally to the daily cycle, including photosynthesis, nitrogen fixation, and gene expression (1–3). Competition assays among different strains of *S. elongatus* rigorously demonstrated that this clock system significantly enhanced the fitness of the cells in rhythmic environments, but not in non-selective constant environments (3). The first circadian rhythm measured in *S. elongatus* was that of *psbAI* promoter activity as assayed by a luciferase reporter in populations of cells (4). More recently, a *tour de force* imaging study visualized the rhythm of luminescence from single bacterial cells (Fig. 1A and B)⁽⁵⁾. That study also demonstrated that as single cells divide, the daughter cells maintain the circadian phase of the mother cell (Fig. 1B). Therefore, the circadian clock in cyanobacteria is not perturbed by cell division. That result confirmed studies in populations of cells that showed the circadian clock ticks away with a period of ~24 h in cells

*Corresponding author: Dr. Carl Johnson Dept. of Biological Sciences, Box 35-1634 Vanderbilt University Nashville, TN 37235-1634 USA TEL: 615-322-2384 FAX: 615-936-0205 Email: carl.h.johnson@vanderbilt.edu.

that are dividing with average doubling times of 6 to 10 h (6^{-8}). Conversely, the circadian clock gates cell division so that there are some times of the day/night cycle when the cells grow without dividing (6). Therefore, two independent timing circuits coexist in this unicellular bacterium; the circadian pacemaker provides a checkpoint for the cell division cycle, but there is no feedback of the cell-division timing circuit upon the circadian clock (8).

Although it was the *psbAI* promoter that was initially found to be robustly rhythmic in *S. elongatus* (4), further investigation of transcriptional control discovered that essentially *all* promoters were modulated by the circadian clock (9). Even a heterologous promoter from *E. coli* is transcribed rhythmically when inserted ectopically into the cyanobacterial chromosome (10). Those observations have now been linked with the discovery that the topology of the entire cyanobacterial chromosome is under the behest of this circadian program. The *S. elongatus* chromosome undergoes robust oscillations of compaction and decompaction that can be visualized with DNA-binding dyes (Fig. 1C)(¹¹). Moreover the superhelical status of DNA experiences correlative circadian oscillations (Fig. 1D)(¹²). Such large-scale changes in chromosomal structure and torsion are likely to modulate transcriptional rates. It is therefore possible that rhythmic gene expression patterns are mediated by daily changes in the topology of the chromosome. From this perspective, the cyanobacterial chromosome might be envisioned as an oscillating nucleoid, or “oscilloid” that regulates all promoters-including heterologous promoters-by torsion-sensitive transcription (12). Gene expression in cyanobacteria is also regulated in a circadian fashion by the putative transcriptional factor RpaA; rhythmic gene expression is attenuated when the *rpaA* gene is deleted (2,¹³). The phosphorylation status of RpaA is regulated by the two-component system kinase SasA, whose phosphorylation is controlled in turn by the KaiABC oscillator that is described in the next paragraph (13). These results support an alternative model in which the SasA/RpaA two-component system mediates signals from the KaiABC oscillator to drive genome-wide transcription rhythms. Although the oscilloid and the SasA/RpaA models appear mutually exclusive, an analysis of stochastic gene expression in cyanobacteria (14) supports regulation both locally (by DNA topology for example) *and* comprehensively (by trans factors such as RpaA).

Cogs and gears: The Kai proteins

The clockwork mechanism that controls these global rhythms of transcription, chromosomal topology, and cell division is composed of three essential proteins-KaiA, KaiB and KaiC-which were identified in 1998 (15). Their three-dimensional structures, which became available in 2004 (16-²¹) are the only core circadian clock proteins for which full-length structures have been determined. KaiA is a dimer of intertwined monomers, KaiB has a thioredoxin-like fold and forms dimers and tetramers, and KaiC is a “double-doughnut” hexamer (See Fig. S1). The structure of KaiC revealed a two-domain fold (N-terminal CI and C-terminal CII lobes) in the monomer and six ATP molecules bound between subunits in both the CI and the CII rings (Fig. 2A). When the three Kai proteins are combined together with ATP in a test tube, a molecular oscillator is reconstituted (Fig. 1E)(²²). This *in vitro* oscillator perpetuates a ~24 h cycle for at least 10 days with KaiC alternating between a hypo-phosphorylated and a hyper-phosphorylated state (23). KaiC is phosphorylated at S431 and T432 residues in the CII half (24,²⁵) (Fig. 3A,B). The CI ring appears devoid of phosphorylation sites and is structurally more rigid than the CII ring. Phosphorylation of CII residues occurs across the subunit-subunit interface, as S431 and T432 are closest to an ATP molecule that is held by the P-loop of the adjacent subunit (Fig. 3A).

KaiC is both an auto-kinase and an auto-phosphatase (26-²⁸) that rhythmically hydrolyzes 15 ATP molecules per subunit during a complete *in vitro* cycle (29). Since only two ATP molecules are needed to phosphorylate S431 and T432, the consumption of the extra ATP

molecules may be used to drive conformational changes within KaiC, including monomer exchange (23,^{30,31}). KaiA promotes the formation of the KaiC hyper-phosphorylated state, while KaiB antagonizes the actions of KaiA and promotes a return to the hypo-phosphorylated state. Structural and biophysical studies have enhanced our understanding of the KaiA•KaiC complex (Fig. 2) (32,³³) and the KaiB•KaiC complex (Fig. 3C,D) (34), as well as quantified the relative levels of KaiC vs. KaiA•KaiC vs. KaiB•KaiC vs. KaiA•KaiB•KaiC complexes formed during the *in vitro* reaction cycle (30,³¹). In addition we now know that the KaiC phosphorylation cycle comprises four consecutive steps: (i) T432 phosphorylation, (ii) S431 phosphorylation, (iii) T432 dephosphorylation and (iv) S431 dephosphorylation (28,³⁵). This information provides the framework for a re-analysis of the Kai protein structures, suggesting how the *in vitro* clock might work.

KaiC interacts with KaiA and KaiB

KaiA binds repeatedly and rapidly to KaiC during the phosphorylation phase (30) and enhances KaiC's auto-kinase activity. Moreover, a single KaiA dimer is apparently sufficient to upregulate phosphorylation of a KaiC hexamer to saturated levels (36). This is consistent with the higher abundance of KaiC *in vivo* relative to KaiA (37). What is the structural basis of KaiA's function? KaiA binds to the KaiC CII lobe C-terminal tail (32). This binding interaction requires concomitant unfolding of an S-shaped loop in the contacted KaiC subunit. We hypothesize that KaiA pulls the S-shaped loop out of the central channel of the KaiC hexameric barrel (Fig. 2). In the KaiC crystal structure the loop residues (aa485-497) are engaged in hydrogen bonding interactions across subunits at the periphery of the channel (Fig. 2B)(²¹). Disrupting the fold of the S-shaped loop of a single subunit might weaken the interface between adjacent CII lobes and promote conformational changes within the CII ring that support phosphorylation at T432 and S431. A three-dimensional electron microscopy (EM) structure of the KaiA•KaiC complex reveals that KaiA is connected to the hexameric barrel of KaiC via a flexible linker (33). The EM structure also suggests that a transient interaction may occur between the apical loop of KaiA and the ATP binding cleft of KaiC. Thus the action of KaiA might be to promote conformational flexibility of the KaiCII ring by disrupting the S-shaped loop hydrogen bond network (Fig. 2C-E) or alternatively, to enhance the residence time of ATP by covering the ATP binding cleft. Therefore, these interactions of KaiA with KaiC could promote the hyper-phosphorylation of KaiC by enhancing (i) its rate of autophosphorylation and/or (ii) its ATP residence time.

The binding mode of KaiB to KaiC differs fundamentally from that of KaiA. Unlike KaiA, which is associated with KaiC during the entire phosphorylation cycle, KaiB preferentially binds to the phosphorylated form of the hexamer (28,^{30,31,35}). Structural analyses combining cryo- and negative-stain EM, X-ray crystallography, native PAGE and fluorescence methods revealed that KaiB dimers bind to the CII ring (Fig. 3C)(³⁴). Instead of interacting with C-terminal KaiC tails, KaiB dimers form a third layer on top of CII without obscuring the central channel. This arrangement has important consequences for KaiA; although still tethered to the C-terminal CII peptide, KaiA is unable to approach the ATP-binding clefts (Fig. 3D). Thus, the EM structure offers a plausible model for KaiB's antagonism to KaiA. In addition to sequestering KaiA, KaiB may use its conserved, negatively charged C-terminal tail to weaken subunit interactions at the CII side of the KaiC hexamer and to destabilize or displace ATP. It is noteworthy in this respect that the EM structure of the KaiB•KaiC complex showed no density for the folded S-loops, suggesting that they are pulled out of the central channel of the KaiC hexameric barrel when KaiB is bound.

Why biological time doesn't run backward

The KaiC crystal structure exhibited double phosphorylation at T432 and S431 in four of the six subunits (21,24). In the remaining two subunits only T432 was phosphorylated (Fig. 3A). The T432 sidechain oxygen atoms are closer on average to the ATP γ -phosphate (7.3Å) than the S431 sidechain oxygen atoms (8.4Å), however neither sidechain is in an optimal position for phosphotransfer. This is not surprising because phosphorylated subunits represent the kinase product state and the phosphorylated sidechains have presumably moved away from ATP to avoid unfavorable electrostatic interactions. The crystal structure shows a subtle 1 to 2 Å variation in the distances between phosphorylation sites and ATP γ -phosphates, suggesting that the CII domains have a tendency to flex. Phosphorylation of T432 results in stabilizing interactions between adjacent CII domains (Fig. 3E, Fig. S2)(24). The phosphate group is engaged in multiple hydrogen bonds to arginine and serine residues, suggesting that local conformational fluctuations will be more limited following the initial phosphate transfer. If S431 is phosphorylated by a pathway similar to that resulting in T432 phosphorylation, the stabilizing intersubunit interactions formed by P-T432 would have to be broken in order to bring S431 in an optimal position for phosphor transfer from ATP. Thus we propose an alternative mechanism for S431 phosphorylation: S431 receives a phosphate from P-T432, followed by immediate rephosphorylation of T432. This alternative mechanism would explain the strict order of phosphorylation events (T432 first and S431 second) and is consistent with the structural data.

Once phosphorylated, S431 can engage in additional hydrogen bonding interactions with amino acids in the same subunit (Fig. 3F, Fig. S3). Among the interacting residues is T426 whose mutation to Ala abolishes clock function (24). The configuration observed in the crystal structure for the T432 and S431 phosphate groups of chain f is particularly interesting in that it shows the two phosphate groups in van der Waals contact and stabilized by an interacting arginine (R393, Fig. 3G). This provides structural evidence that T432 and S431 can in principle get close enough to each other to allow for a phosphate transfer and supports the proposed alternative phosphorylation mechanism. Another structural observation supporting the alternative mechanism is that in the two subunits exhibiting phosphorylation only at T432 (chains c and d; Fig. 3A), the sidechain oxygen atom of S431 lies closer to the phosphate of P-T432 (7.0Å) than to the γ -phosphate of ATP (8.4Å). Overall, the structural information on the phosphorylation events at the KaiCII subunit interfaces and the inter- and intra-subunit interactions formed by the phosphorylated residues indicates that the number of hydrogen bonds increases as first T432 and subsequently S431 is phosphorylated. This progressive increase in molecular interaction would make the reverse reactions unfavorable, causing a built-in ratcheting mechanism that drives the KaiC oscillator unidirectionally.

We envision that a conformational change is then required to drive KaiC forward to the phosphatase state and achieve dephosphorylation first of T432 and then of S431 in all six subunits. Sequential dephosphorylation in this order has been observed in biochemical assays (28,35). Interaction of KaiB with KaiC facilitates the formation of the phosphatase state. Unlike KaiA, which has similar affinities for various forms of KaiC (30), KaiB binds preferentially to the hyper-phosphorylated form of KaiC (specifically, P-S431; 28,35). KaiC can thus be either a kinase or a phosphatase; at the present time, only the kinase state has been captured in a high-resolution crystal structure.

How does this in vitro clockwork tick?

The unexpected demonstration that KaiC's phosphorylation status continued to cycle when the three Kai proteins are combined in a test tube and ATP was added to provide energy (Fig. 1E) (22), shows that circadian oscillations are not absolutely dependent upon transcriptional and/

or translational feedback (22,27,38,39). The *in vitro* rhythm, KaiC's dephosphorylation rate, and KaiC's ATP hydrolytic activity are all temperature compensated (22,29,39), meaning that a temperature compensation mechanism is intrinsic to the characteristics of the three Kai proteins and the nature of their interactions. How this is accomplished is an important outstanding question (22,29,39).

The availability of the *in vitro* system for analyzing the molecular nature of a circadian clockwork allows biophysical, biochemical, and structural analyses that were previously impossible. Electron microscopy, chromatography, and native gel electrophoresis techniques have been applied to quantify the time dependent formation of complexes among the Kai proteins (30,31). KaiC exists in all possible combinations with KaiA and KaiB throughout the *in vitro* oscillation: free KaiC hexamers, KaiA•KaiC complexes, KaiB•KaiC complexes, and KaiA•KaiB•KaiC complexes (Fig. 1E). The proportions of these complexes vary in a phase-dependent manner: free KaiC hexamers predominate at all phases, ~10% of KaiC hexamers appear as KaiA•KaiC complexes at all phases, and KaiB•KaiC and KaiA•KaiB•KaiC complexes are clearly rhythmic, becoming most common in the KaiC dephosphorylation phase (Fig. 1E)(30,31). Therefore, during the *in vitro* oscillation, KaiC is undergoing rhythmic changes in conformation, phosphorylation status, and interactions with KaiA and KaiB.

Maintenance of a high amplitude oscillation in the face of noise is a crucial characteristic of any circadian oscillator (5). In the case of the *in vitro* oscillator, KaiC monomers exchange among the hexamers, a process that synchronizes the phosphorylation status of the individual hexamers in the population of hexamers (Fig. 4; refs. 23,30,31). Consequently, although cyanobacterial cells in populations are autonomous oscillators that do not communicate phase information intercellularly (5), communication among KaiC hexamers via monomer exchange maintains a high-amplitude rhythm inside the cell (23,31). Therefore, the post-translational cyanobacterial clockwork is composed of biochemical reactions such as phosphorylation, ATP hydrolysis, monomer exchange, and conformational changes among thousands of molecules per cell (~10,000 KaiC monomers/cell)(37), permitting robust oscillations of high precision and synchrony.

From test tube to cell; embedding the KaiABC oscillator within a TTFL

What is the role of the post-translational KaiABC oscillator in the overall cellular program (Fig. 4)? There is a rhythm of KaiC phosphorylation *in vivo* that continues in the absence of transcription or translation, but there are also rhythms of KaiB and KaiC abundance in metabolically active cells that have been interpreted in terms of a TTFL (39,40). Perhaps the rhythms of KaiC phosphorylation and abundance are complementary processes that can oscillate independently or can interact to generate a more robust overall program (41). Early evidence for a core TTFL oscillator in cyanobacteria was in part based on experiments in which KaiC abundance was experimentally manipulated *in vivo*; KaiC expression from an inducible promoter reset the cellular clock in a phase-dependent fashion (15,40). While the most direct impact of KaiC expression is upon abundance, such expression could also perturb the phosphorylation oscillator; flooding KaiC pools with newly synthesized and therefore unphosphorylated KaiC would be expected to alter the phosphorylation ratio of the KaiC pool. If the new synthesis of KaiC occurs at a phase when hexamers are predominantly hypophosphorylated, the oscillation of KaiC phosphorylation would be reinforced (enhanced amplitude). On the other hand, new synthesis of unphosphorylated KaiC when hexamers are predominantly hyper-phosphorylated would lead to an overall decrease in the KaiC phosphorylation status, thereby altering the phase of the KaiABC oscillator (phase shift) and/or reducing its amplitude. Therefore, the post-translational oscillator (PTO) may regulate the timing of transcription/translation to occur in an optimal phase to enhance robustness of the larger oscillating system (Fig.4). In this scenario, the PTO is embedded in a TTFL; the PTO

may most directly determine the dynamics of the circadian system but the TTFL provides a secondary feedback loop that aids robustness.

What are the potential benefits of a biochemical (PTO) oscillator embedded within a genetic (TTFL) oscillator? A core oscillator that is composed of biochemical reactions among thousands of molecules per cell should be more robust in the face of metabolic noise than one founded on transcriptional activity. This is particularly true for cells that must maintain precise timekeeping during cell division, when the ratio of DNA to transcriptional factors can change during replication and where DNA can become less accessible when chromosomes condense in preparation for division. The advantage provided by a biochemical oscillator such as KaiABC is that this post-translational system would be less susceptible to the influences of cell division (5⁻⁸) or major changes in metabolic rate (39,40) than one based on transcriptional and translational rates. Although eukaryotic circadian genes are not homologous to *kaiABC* sequences, the proteins they encode also undergo circadian rhythms of abundance and phosphorylation (1,42,43). The benefit of a clockwork that is imperturbable even when buffeted by the massive intracellular changes of cell division could have provided an evolutionary driving force for convergent circadian clock mechanisms among diverse organisms.

We now recognize KaiABC as a dynamically oscillating nanomachine that has evolved to precess unidirectionally and robustly. The challenges ahead are to delve deeper into the molecular nature of its temperature compensation, to examine the place of the PTO in the larger cellular program, and to discover if the clocks in our own cells have attributes that are similar to those of bacteria.

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Fig. 1. Rhythms in cyanobacteria from cells to molecules

(A and B) Circadian rhythms of bioluminescence in single cyanobacterial cells. Panel A shows micrographs of cyanobacterial cells at different times in constant light conditions—upper panel is the brightfield images showing growth and cell division as a function of approximate circadian time, lower panel is the luminescence emanating from these cells (the luminescence reporter was the *psbAI* promoter driving expression of bacterial luciferase, *luxAB*). Panel B shows the quantification of bioluminescence from a single cell as it divides into multiple cells as a function of time in constant light; starting at day 1.5, there are two differently colored traces as a result of cell division, the next division occurs at day 2.0, and so on (panels A and B are courtesy of I. Mihalcescu, reprinted with permission from (5)). (C) Circadian rhythm of

chromosomal compaction as visualized by a fluorescent DNA-binding dye (green)(red is chlorophyll autofluorescence). The chromosome is more compacted in the subjective night (hours 12-20) and less compacted in subjective day (hours 0-8, 24-28)(panel C is courtesy of S. Williams, reprinted with permission from (11)). (D) Chromosomal topology shows a circadian rhythm as assayed by supercoiling of an endogenous plasmid. Topoisomers of the plasmid are more relaxed (R) in the subjective night and are more supercoiled (SC) in the subjective day (12). (E) KaiC phosphorylation in the oscillating *in vitro* reaction is shown by SDS PAGE in the upper panel; upper bands are hyper-phosphorylated KaiC, lower band is hypo-phosphorylated KaiC. The lower panel shows the predominant species of complexes of KaiA, KaiB, and KaiC that form during the *in vitro* oscillation: hypo-phosphorylated and uncomplexed KaiC hexamer (lower row) is present at all phases, but KaiC in complexes with KaiA and/or KaiB form rhythmically in concert with changes in KaiC's phosphorylation status (upper line, only the predominant complex is shown). Light blue KaiC hexamers are in the phosphorylating phase prior to monomer exchange, while dark blue KaiC hexamers are those undergoing dephosphorylation and monomer exchange.

Fig. 2. KaiA-KaiC interaction

(A) The crystal structure of the KaiC hexamer shows a double doughnut shape formed by two lobes per subunit (PDB-ID 2GBL). The S-shaped loops (aa485-497)(green) dip into the central channel of the hexameric barrel. The flexible C-terminal residues (aa498-519) extend from the CII end of the hexamer. Some of the C-terminal tails are shorter than others because this region is partly disordered and only two chains have been completely traced out to the C-terminal S519 residue. ATP (gold) is bound between subunits in both the lower CI ring and the upper CII ring. (B) The six S-shaped loops (green stick representation) interact via a hydrogen bond network. Hydrogen bonds are shown in magenta and the view is perpendicular to (A) as seen from the CII side. (C) The CII end of one KaiC monomer as in the crystal structure with the S-shaped loop in green. (D) The CII end of one KaiC monomer as proposed to interact with KaiA, with the S-shaped loop pulled out (33). In parts (C) and (D) the S-shaped loop and C-terminal tail are shown as a wide ribbon. (E) Model of the KaiA-KaiC interaction based on combined structural information from x-ray crystallography, NMR, and three-dimensional electron microscopy (33). One S-shaped loop (green) is shown pulled out of the KaiC hexameric barrel. For clarity the S-shaped loop and C-terminal residues are only shown for one of the six KaiC subunits. The KaiA dimer is shown in red and purple.

Fig. 3. Structural stabilization effects of phosphorylation and KaiB-KaiC interaction

(A) Ring of CII lobes from the KaiC crystal structure. Four subunits, chains a, b, e, and f (pink), are doubly phosphorylated at S431 and T432. Two subunits, chains c and d (blue), are singly phosphorylated at T432. The sidechains of S431 and T432 are shown in space filling representation and are colored by element with phosphorus atoms in cyan and oxygen atoms in red. ATP (gold) is bound between the CII lobes. (B) CII lobe of a doubly phosphorylated KaiC monomer (chain a) with the S-shaped loop in green. The view is perpendicular to (A). (C) Model of the KaiB•KaiC interaction based on combined structural information from x-ray crystallography and three-dimensional electron microscopy (34) with KaiB dimers in green and gold. (D) Model of the KaiA•KaiB•KaiC interaction with KaiA (red) and KaiB (green) dimers in orientations resembling those in the class IV KaiABC particle images from negative-stain EM (see Fig. 1D in ref. 31). (E) Hydrogen bonds (green) formed by the phosphate group of T432 in chain c (blue). (F) Hydrogen bonds formed by the phosphate groups of S431 and T432 in chain b (pink). In both (E) and (F) the neighboring chain is shown in gray. See Figs. S2 and S3 for the hydrogen bonds formed by the additional phosphate groups in chains a-f. (G) In chain f the phosphate group of S431 is leaning toward T432, rather than toward T426 as in chains a, b, and e. The position of the S431 phosphate group in chain e is shown in a faint representation. The phosphate groups of both S431 and T432 in chain f are stabilized by electrostatic interactions with R393.

Fig. 4. A self-sustained post-translational oscillator (PTO) embedded within a transcription and translation feedback loop (TTFL)

The post-translational KaiABC oscillator (cycle connected by red arrows) is determined by phosphorylation of KaiC (blue hexamers) as regulated by interactions with KaiA (red dimers) and KaiB (green tetramers). Robustness is maintained by synchronization of KaiC hexameric status via exchange of KaiC monomers (23,30,31). Monomer exchange is depicted in the center of the PTO by KaiC monomers exchanging with KaiC hexamers; phase-dependent changes in the rate of monomer exchange is indicated by the thickness of the double-headed black arrows. New synthesis of KaiC feeds into the KaiABC oscillator as non-phosphorylated hexamers or as monomers that exchange into preexisting hexamers. The PTO brings KaiC to a state that regulates chromosome topology and/or transcriptional factors (“TFs”) to control global transcription of all promoters (including those driving expression of the essential clock genes *kaiA*, *kaiB*, and *kaiC*).