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# Orchestration of circadian timing by macromolecular protein assemblies

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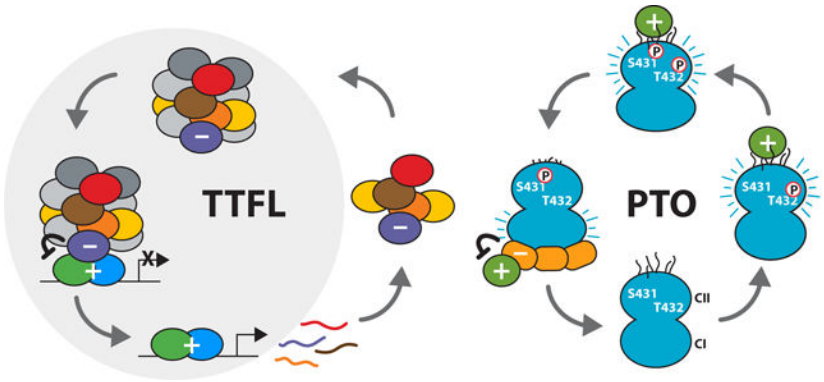
## Abstract

Genetically-encoded biological clocks are found broadly throughout eukaryotes and in cyanobacteria, where they generate circadian (*about a day*) rhythms that allow organisms to anticipate regular environmental changes and align their physiology and behavior with Earth's daily light/dark cycle. In recent years, many have sought to expand our biochemical and structural understanding of the clock proteins that constitute the molecular 'cogs' of these biological clocks. These new studies are beginning to reveal how macromolecular assemblies of dedicated clock proteins form and evolve to contribute to the generation of clocks that function over the timescale of a day. This review will highlight structural and biochemical studies that provide important insight into the molecular mechanisms of cyanobacterial and vertebrate animal clocks. Collectively, these studies demonstrate emerging biochemical properties that appear to be shared by these different clocks, suggesting that there may be some conservation in the regulation and assembly of circadian macromolecular assemblies.

## Graphical Abstract

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## Introduction

Circadian rhythms help organisms anticipate and adjust to changing environmental conditions in a world that is temporally programmed on a 24-hour schedule by the rotation of Earth about its axis. Daily rhythms arise from genetically-encoded molecular clocks that originate at the cellular level and operate with an intrinsic period of about a day. By definition, the timekeeping encoded by these self-sustained clocks must persist in constant conditions (i.e., the absence of a light/dark cycle) but respond to environmental cues, or zeitgebers (*time-givers*), like light to align internal clocks with the external environment [1]. Therefore, circadian rhythms help an organism predict regular changes in their environment and program behavior and physiology to occur at the right time of day [2], whether it be the coordination of activity, appetite, and metabolic processes [3], or the execution of widespread programs of DNA repair to maintain genomic integrity [4, 5].

At the molecular level, circadian rhythms are generated by delayed negative feedback loops that create robust oscillations in gene expression and/or protein activity with a period of about a day [6]. Resetting cues, known as zeitgebers (*time-givers*), adjust the phase of these oscillations to align them with external stimuli, linking biology to Earth's 24-hour terrestrial day [7]. Two mechanisms give rise to circadian rhythms at the molecular level: the transcription/translational feedback loop (TTFL) and the post-translational oscillator (PTO) (Figure 1). Of these two, the TTFL is the most common molecular architecture that underlies circadian rhythms. In a TTFL, transcription factors form the positive arm of the feedback loop and induce the expression of genes that constitute the negative arm; after a delay, repressor proteins of the negative arm feedback to interact with or modify the transcription factors to repress their activity, thus generating an oscillation in gene expression with a period of about a day for circadian rhythms [8]. The central timekeeping functions of eukaryotic clocks from green algae and plants to fungi, insects and humans are all built upon TTFLs that use different genes for the positive and negative components but follow the same overall feedback loop structure [9].

The cyanobacterial circadian clock serves as the paradigm for a post-translational oscillator that is generated exclusively from the biochemical activity of clock proteins. In a PTO, the delayed negative feedback loop is generated through rhythmic changes in protein conformation, enzymatic activity and/or post-translational modifications that are regulated by changes in protein complexes formed throughout the day [10]. Remarkably, the cyanobacterial clock can be reconstituted *in vitro* with three purified Kai (*cycle*) proteins, $Mg^{2+}$, and ATP as an energy source [11]. In the cyanobacterial circadian PTO, autophosphorylation of the central clock component KaiC is activated during the day by KaiA and inhibited at night by KaiB. However, it's important to note that although this PTO can function *in vitro* in the absence of transcription, it is embedded within a TTFL *in vivo* that is important for the overall robustness and phase resetting of the clock [12, 13].

One other circadian PTO has been identified in recent years; this PTO generates rhythms of protein oxidation that occur on a daily timescale and persist in isolated red blood cells that are enucleated and incapable of transcription [14]. The same circadian protein oxidation rhythms have also been observed in cyanobacteria and green algae, plants, fungi and higher eukaryotes that all have TTFLs [15, 16], suggesting that circadian PTOs like this could represent ancient mechanisms to encode circadian signaling at the biochemical level [17].

With overt differences in the molecular architecture of circadian clocks based on TTFLs or PTOs (i.e., transcription-based or transcription-independent), it's not immediately obvious that they might share some similarities in key molecular steps that regulate the generation of biological rhythms on such a long timescale. For instance, although TTFLs can leverage the time it takes for transcription, post-transcriptional regulation and translation to build several hours-long delays into the ~24-hour-long feedback loop of the molecular clock, a growing body of evidence suggests that vertebrate TTFLs also rely heavily on the slow accumulation of regulatory post-translational modifications and the formation and remodeling of large clock protein complexes [18–20]. Recent biochemical and structural studies now show that macromolecular complexes of the vertebrate circadian clock are also regulated by competition for mutually exclusive binding interfaces [21–25] just like those found in the cyanobacterial PTO [26–29]. This review will focus on the molecular details of clock protein interactions and how regulation of their macromolecular protein assemblies is thought to contribute to circadian timekeeping in cyanobacteria and vertebrates.

## The Kai protein PTO in cyanobacteria

The remarkable discovery that the cyanobacterial circadian clock can function as a posttranslational oscillator was first made by Kondo and colleagues in 2005 through reconstitution of self-sustaining ~24-hour phosphorylation rhythms in the hexameric AAA+ ATPase KaiC that are regulated by interaction with KaiA, KaiB, $Mg^{2+}$ and ATP *in vitro* [11, 30]. The KaiC protein has two ATPase domains, CI and CII, that are connected by a short linker. Circadian rhythms in phosphorylation of KaiC occur at two neighboring residues in the C-terminal CII domain on S431 and T432 [31, 32]. Once the PTO was reconstituted *in vitro*, the biochemical basis for the sequential phosphorylation cycle of KaiC was established soon thereafter [33, 34]. KaiC begins each day in an unphosphorylated state at dawn, followed by slow autophosphorylation first on T432, and then on S431 to create the doubly phosphorylated pSpT species by dusk (Figure 1B). From this point of peak phosphorylation, KaiC begins to trigger its own slow dephosphorylation via a phosphotransfer mechanism similar to that of ATP synthase [35, 36], acting first on T432 to create the key nighttime pST output signaling state [37], followed by dephosphorylation of S431 to begin the cycle again.

KaiC is the central enzymatic driver of the cyanobacterial clock, but it requires the other two Kai proteins to establish a robust and self-sustained PTO [11, 38]. KaiA acts in the positive arm of the PTO feedback loop by binding to C-terminal extensions of KaiC known as the A-loops [27, 39, 40] that are reversibly extended in the day (Figure 1B) [41, 42]. Phosphorylation of T432 on KaiC during the day destabilizes the CII ring to favor A-loop extension [43, 44] in a process that is coupled to ATP hydrolysis and nucleotide release

to control the conformation of the CII hexameric ring [45, 46]. Overall, the release of KaiC A-loops allows KaiA to act as a nucleotide exchange factor [47], accelerating the intrinsically slow release of ADP by KaiC and promoting the uptake of ATP [48]. The ability to stimulate KaiC autophosphorylation during the day, when the [ATP]/[ADP] ratio is at its highest due to photosynthesis, helps the clock to entrain to the light/dark cycle through light-driven metabolic changes [49, 50]. Once KaiC reaches its doubly phosphorylated state at dusk, the repressor KaiB binds directly to KaiC [34]. Generation of the subsequent pST state on KaiC further stabilizes the CII ring to induce retraction of the KaiA-binding A-loops [42, 43] and enhance formation of a KaiABC ternary complex that sequesters and inactivates KaiA [28, 29], allowing KaiC to slowly autodephosphorylate throughout the evening and reinitiate the cycle in the morning.

## Structural basis for clock protein assembly in cyanobacteria

How do the three Kai proteins interact with one another throughout the day to generate this PTO? Structures of the individual Kai proteins were published many years ago, even before the post-translational nature of the cyanobacterial oscillator came to light. These structures revealed that the KaiC hexamer has a characteristic double-donut shape similar to other AAA+ ATPases [51], KaiA exists as an α-helical domain-swapped dimer flanked by N-terminal pseudoreceiver (PsR) domains [52], and KaiB takes on a tetrameric ground state as an apparent ‘dimer of dimers’ [53] (Figure 2). A close integration of structural, biophysical, and computational studies with biochemical analyses of the cyanobacterial clock *in vitro* have helped to reveal how KaiA binds to KaiC during the day to fulfill its role as the positive arm of the PTO. Rather than make a direct association with the ATPase domains of KaiC, KaiA binds an α-helical A-loop extension from one subunit of KaiC into a cleft at its α-helical dimer interface (Figure 3A) [27, 39, 40]. MD simulations show that A-loop extension and nucleotide release are coupled by a conformation change at the intersubunit interface in the CII domain [45], providing a mechanistic framework to explain how KaiA acts as a nucleotide exchange factor for KaiC.

The molecular basis for KaiB binding to KaiC and formation of the repressive KaiABC ternary complex remained unclear for more than a decade after the initial reconstitution studies. Early attempts to structurally characterize complexes of KaiB and KaiC by electron microscopy were limited by the low resolution of the technique at the time, although it was clear that KaiB formed a ring-like structure on one of the hexameric faces of the KaiC [54–56]. Since KaiB binding is strictly regulated by the phosphorylation state of S431 in the KaiC CII domain [34, 57], the first model suggested that KaiB docks in close proximity to this site on the CII domain [54]. However, this initial model eventually conflicted with biochemical experiments dissecting the molecular determinants of KaiB binding, which consistently demonstrated that it bound directly to the CI domain of KaiC [42, 43, 58]. This second model, that KaiB binds at the hexameric face on the CI face of KaiC, was also consistent with observations that CI ATPase activity is required for KaiB binding [57] and that ATP hydrolysis in the CI domain leads to a series of conformational changes throughout the CI domain [59] that terminate at the B-loops, a CI-specific insertion that is solvent accessible on the face of the hexamer and required for KaiB binding [42].

The breakthrough that ultimately resolved this question came from LiWang and colleagues in 2015 with their discovery that KaiB undergoes a massive structural reorganization from its tetrameric ground state to an active KaiC-binding monomer state [26]. This dramatic change in the KaiB structure was initially suggested by NMR spectroscopy based on extensive changes in the backbone chemical shifts of KaiB upon formation of a stable complex with an engineered CI domain monomer from KaiC [26]. Studies eventually revealed that the C-terminal half of KaiB that makes direct contact with KaiC takes on a completely new fold with a previously buried β-strand becoming a solvent accessible α-helix and vice versa (Figure 3B). In this regard, KaiB joins a relatively rare but growing class of proteins that exhibit similar metamorphic properties [60]. Other metamorphic proteins use changes in the local environment (e.g., ionic strength or hydrophobicity), post-translational modifications, or interactions with other proteins to alter their intrinsic fold-switching equilibria [61–63]. None of the backbone chemical shifts that provide a signature for the fold-switched state are visible in NMR spectra of native KaiB, suggesting that this fold is very rarely populated under standard experimental conditions in the absence of KaiC [26]. The LiWang lab used the chemical shift ‘fingerprint’ of the fold-switched protein to identify a series of structure-based mutations in KaiB that stabilized this active monomeric state, facilitating its biochemical characterization as a high affinity binding partner of KaiC [26, 29]. Critically, locking KaiB permanently into the fold-switched state disrupts the PTO *in vitro* and circadian rhythms in *Synechococcus elongatus* because it traps KaiC in its nighttime repressive state [26]. These findings suggest that the regulatory mechanisms that govern KaiB fold-switching and its interactions with KaiC are likely to be very important for cyanobacterial circadian rhythms.

We now have a complete structural accounting of Kai protein interactions, including two structures of the ternary KaiABC complex that forms in the repressive phase of the PTO at night. Two different approaches were taken to visualize the KaiABC complex: the first utilized a mutant of KaiB locked into the fold-switched conformation to nucleate formation of a stable, homogeneous KaiABC complex that was determined by x-ray crystallography [29], while the second took advantage of the observation that KaiC phosphorylation is favored at 4°C [33] and that KaiA, B and C spontaneously assemble into complexes with prolonged incubation at cold temperatures; with the use of single-particle cryo-electron microscopy, it was possible to solve a structure of the KaiABC complex out of this heterogeneous mixture [28]. While the crystallographic structures used engineered proteins (fold-switch ‘locked’ KaiB and truncated versions of KaiA and KaiC), they provided detailed, high-resolution snapshots of the key interactions that mediate complex formation (Figure 3C); by contrast, the electron microscopy study used native proteins for structure determination, but only reached ~5 Å resolution to provide an overall image of the fully assembled KaiABC complex [28]. Despite these different approaches, both structures converged to reveal the same interdomain contacts between Kai proteins. Critically, the EM structure captured native KaiB in its fold-switched conformation in this complex [28], further validating that KaiB dynamically refolds from the ground state structure observed in its apo state as a tetramer to the fold-switched monomer observed in the presence of KaiC [26].

By revealing the structural basis for the KaiB-KaiC interaction, these studies also provided a framework to begin to understand the pivotal role that KaiC ATP hydrolysis plays in the clock. In addition to the exceedingly slow rate of autophosphorylation that occurs in the CII domain [11, 33, 34], the CI domain of KaiC catalyzes hydrolysis of ~10–12 ATP per subunit per day [59]. Remarkably, mutations that alter the rate of ATP hydrolysis in the CI domain are highly correlated with the period of the cyanobacterial PTO *in vitro* and in *Synechococcus elongatus* [59, 64]. Several crystal structures by Akiyama and colleagues demonstrate how ATP hydrolysis in the CI domain induces a series of local conformational changes that terminate at the KaiB-binding B-loop of KaiC [42, 59]. Given that CI ATPase activity is necessary for KaiB binding [57], which forms the basis for assembly of the macromolecular complex that inhibits KaiA [28, 29], these data suggest that ATP hydrolysis may play a role in the rate-limiting assembly of the repressive KaiABC complex to define the period of the cyanobacterial PTO.

## Competition for mutually exclusive binding sites in the cyanobacterial clock

Perhaps the most exciting revelation from the structures of cyanobacterial proteins and their macromolecular assemblies is the recurring observation that clock proteins compete with one another for overlapping binding sites. The precedence for regulation of protein function on the basis of competition for mutually exclusive binding sites exists in diverse signaling pathways [65, 66]. Moreover, these competitive mechanisms can make the conversion between active to inactive states of a protein more ultrasensitive (i.e., switch-like) instead of graded [67], and switch-like responses have been shown to amplify rhythms in molecular circadian clocks and prevent them from reaching an equilibrium that terminates oscillation [68]. Therefore, understanding the structural basis for competitive and cooperative interactions could shed light on mechanisms that are particularly important for oscillatory networks like circadian clocks. Several examples of competitive interactions in the cyanobacterial clock based on recent structures and biochemical data are briefly outlined below.

Both structures of the KaiABC ternary complex revealed a dramatic change in the conformation of the KaiA dimer from the open state observed in its apo structure (Figure 2A) to a closed state characterized by a large rotation of the β6 strand that links its C-terminal α-helical dimer to the N-terminal PsR domains [28, 29]. The rotation of β6 into this position is stabilized by a direct interaction with KaiB in the KaiBC complex, which then places the adjacent α5 helix into the same binding cleft used to bind the A-loops of KaiC (Figure 4A) [27, 39, 40]. Therefore, binding to the KaiBC complex induces an autoinhibited conformation in the KaiA dimer that effectively eliminates its ability to stimulate autophosphorylation by KaiC. Mutations on the α5 helix of KaiA that disfavor the closed, autoinhibited state disrupt circadian rhythms *in vivo*, demonstrating the importance of the structural switch observed here [29]. Therefore, the efficient sequestration of inactive KaiA into the nighttime KaiABC repressive complex defines an important molecular step in the cyanobacterial PTO.

A detailed analysis of the high-resolution crystallographic structure of the KaiABC complex further showed that KaiA associates with the KaiBC complex through a cooperative network

of interactions with both KaiB and KaiC [29], although interactions with KaiB are required to recruit KaiA to this macromolecular assembly [42]. Notably, KaiA uses its β6 linker strand to form an intermolecular β-sheet with KaiB by docking into the exposed β2 strand of KaiB—the same interface used by the PsR domain of the clock protein CikA to dock onto the KaiBC complex (Figure 4B) [29]. The histidine kinase CikA serves an enigmatic role as both an input and output protein for the cyanobacterial clock [69]. Recruitment of CikA to the KaiBC complex via its PsR domain activates its intrinsic phosphatase activity towards the response regulator RpaA to facilitate transcriptional output via regulation of clock-regulated genes *in vivo* [70]. However, the PsR domain of CikA also binds to oxidized quinones that serve as an acute input signal of darkness to the clock [71–73]. While the molecular details of resetting by this zeitgeber are not fully understood yet, the binding of oxidized quinones to the CikA PsR domain may alter its interaction with the KaiBC complex to facilitate phase resetting of the clock. The ability of the CikA PsR domain to compete with KaiA for the same binding site on KaiB in the nighttime repressive complex with KaiC may explain why the addition of this domain shortens circadian period in the cyanobacterial PTO [26]—by releasing KaiA from its autoinhibited state in the KaiABC complex, the CikA PsR appears hastens the end of the repressive phase and allow KaiC to reinitiate its cycle of autophosphorylation [74]. Therefore, competitive interactions on the KaiBC complex at night likely play important roles in maintaining the length of the repressive phase of the cyanobacterial PTO.

Finally, evidence from biochemical assays demonstrates that KaiB also competes directly with the clock output protein SasA for the same binding site on the B-loops of the KaiC CI domain [42, 55]. Recruitment of the histidine kinase SasA to the pSpT state of KaiC at dusk is required to activate its kinase activity towards the response regulator RpaA to regulate downstream gene expression [70]. Competitive interactions presumably come into play when both SasA and KaiB attempt to bind the CI domain of KaiC, which could affect SasA-dependent output and/or assembly of the KaiBC complex [42, 43, 55, 75]. Unlike KaiA and CikA, which dock onto the same binding site on KaiB but with different domains [29], KaiB and SasA likely use the same protein fold to bind to the B-loop on the KaiC CI domain (Figure 4C) [26, 42, 43]. Based on a high degree of sequence conservation, it was initially assumed that KaiB and the N-terminal domain of SasA would have similar structures [76]. However, when the NMR structure of the SasA N-terminal domain was first determined in 2004, it revealed a thioredoxin-like fold that was quite different from the first published structure of KaiB, which crystallized as a dimer in what we now know to be the canonical ground state conformation [76, 77]. The LiWang lab's groundbreaking discovery in 2015 that KaiB takes on a thioredoxin-like structure in its active, fold-switched monomer state [26] provides a conceptual framework to begin to explore the functional significance of competition between KaiB and SasA. Moreover, this work provides a clear path forward to investigate how the balance of KaiB sequestration into an inactive ground state oligomer influences its stabilization by KaiC into the active KaiB monomer fold, and how competitive interactions with the other clock proteins defined here contribute to the circadian timekeeping mechanisms of cyanobacteria.

## A clock conundrum—how is the KaiC phosphocycle linked to KaiB binding?

One of the major unresolved questions in the cyanobacterial PTO is identification of the molecular basis by which information about the KaiC phosphorylation cycle in the CII domain is allosterically communicated to the CI domain to regulate CI ATPase activity and KaiB binding. Biochemical studies of isolated CI and CII hexameric rings demonstrate that the doubly phosphorylated pSpT and subsequent pST states of the CII ring sequentially increase its rigidity to allow it to 'stack' onto the CI ring [43, 78], suggesting that the CII phosphostate could be allosterically communicated to the CI domain. This observation is consistent with solution biophysical studies of full-length KaiC by small-angle x-ray scattering (SAXS) and tryptophan fluorescence that suggest a change in the overall conformation of KaiC in its nighttime phosphorylation state [79, 80]. However, this conclusion is at odds with crystal structures of various KaiC phosphomimetic mutants, which demonstrate no substantial structural differences in intersubunit packing within a hexamer ring or in the association of the tandem ATPase domains [51, 81] despite the fact that phosphomimetic mutants appear to faithfully recapitulate the phase-dependent biochemical properties of KaiC (e.g., the preferred interaction of KaiB with the nighttime state is recapitulated with the S431E/T432E or S431E/T432A mutants) [64, 80].

It's not clear whether the structural similarity of phosphomimetic KaiC mutants in the crystal structures arises from the use of non-hydrolyzable ATP analogs that lock KaiC into an inactive state or whether crystal packing interactions stabilize similar conformations. The structure of the S431E phosphomimetic KaiC hexamer (locked permanently into the nighttime state) crystallized with KaiB in the presence of ATP was able to capture the CI domain in the post-ATP-hydrolysis state, fully bound by six fold-switched monomers of KaiB (Figure 4D) [29]. However, the resolution of this structure (3.8 Å) and apparent absence of bound nucleotide in the CII domain limited insight here, and interdomain stacking interactions observed were largely the same as prior structures [29, 51, 81]. Identifying the molecular basis for communication through the KaiC hexamer will provide critical mechanistic insight into the cyanobacterial PTO, since numerous experimental and computational models have linked the pS phosphorylation state with allosteric communication through the KaiC protein [82, 83].

While the structure of the hexameric KaiBC complex does not yet appear to address the intersubunit interactions involved in communicating information across the two ATPase domains of KaiC, it does help to provide a rationale for the cooperative binding of KaiB observed by native mass spectrometry [28, 84, 85]. In addition to its contacts with the B-loop of KaiC, each KaiB monomer makes substantial contacts with its neighboring KaiB subunits to create a highly stable hexameric complex in the presence of KaiC [29]. Notably, a mutation localized to one of the KaiB-KaiB interfaces between adjacent monomers significantly reduces the apparent affinity of KaiB for KaiC [77], demonstrating what is likely to be an important role for cooperative assembly of KaiB into the KaiC hexamer. Future studies to elucidate the role for cooperative assembly of KaiB onto KaiC should illuminate yet another key point of control for circadian timekeeping in cyanobacteria.

## Towards a real-time characterization of macromolecular assemblies in the cyanobacterial PTO

What remains on the horizon for the cyanobacterial PTO? Although tracking the KaiC phosphorylation cycle by electrophoretic methods [11] provided the foundation for detailed biochemical studies that have nearly laid out the complete mechanistic basis for this molecular clock, the development of new techniques to probe other aspects of this PTO offers exciting opportunities to researchers in this field. For example, there are now methods that allow real-time tracking of Kai protein interactions *in vitro* using fluorescence polarization (FP) (Figure 4E) [86] and in the cellular context using fluorescence correlation spectroscopy [87]. Aside from its obvious benefits of scalability to 96 or 384-well plates, the FP-based assay offers an advantage over time-consuming sampling and electrophoretic characterization of KaiC phosphostates that could be applicable for large-scale mutagenesis screens [88], all while providing continuous, real-time information on macromolecular assembly of KaiABC complexes non-invasively.

## Dissecting the molecular basis of the prototypical TTFL from vertebrate circadian clocks

Moving from the relatively ‘simple’ cyanobacterial PTO to a description of the molecular architecture of a transcription/translation feedback loop (TTFL) requires an adjustment of scope, as far less is known about the molecular details of TTFLs. The obligate inclusion of a circadian cycle of transcription, RNA processing and translation means that clocks based on TTFLs must in some way engage with and depend on general cellular machinery for transcription, post-transcriptional regulation and translation. Consistent with this, a genome-wide siRNA screen in human cells for modifiers of the vertebrate circadian clock identified an inordinate number of genes that contribute to circadian period and the amplitude of cycling [89]. For the purposes of this review, I will focus on the protein structures and biochemical determinants of the clock proteins involved in the core TTFL of the vertebrate clock that regulate their assembly into macromolecular complexes. There has generally been two approaches to generate molecular insights at this level in the vertebrate circadian clock: the first is based on classical, biochemically-based bottom-up strategies that dissect key protein-protein interactions from high-resolution structures of these components (reviewed recently in [90]), and the second leverages a top-down approach to purify and characterize components of native clock protein complexes (e.g., as in [18, 91]). Our understanding of the vertebrate circadian clock has benefitted from these different approaches that collectively provide an overview of the biochemical complexity at hand as well as a more granular understanding of the molecular basis by which clock protein assemblies come together.

## Evidence for several distinct temporally-regulated clock protein complexes on chromatin

Nearly two decades ago, Reppert and colleagues published an encyclopedic catalog of clock protein interactions and associated post-translational modifications that serve as the foundation for our current understanding of the core TTFL of the vertebrate circadian clock [19]. In this initial model, the circadian transcription factor CLOCK:BMAL1 binds to conserved E-box elements in the promoters of its repressor genes, *Period* (*Per*) and *Cryptochrome* (*Cry*) to drive their transcription each morning. PER1 and PER2 interact with one another, the cryptochromes (CRY1 and CRY2), and the dedicated clock kinases,

Casein Kinase 1δ and/or CK1ε, to form a macromolecular assembly of repressor proteins. After translocation to the nucleus in the evening, this repressor assembly binds to CLOCK:BMAL1 and inhibits transcriptional activation. The cycle presumably begins again as the repressor proteins are degraded and CLOCK:BMAL1 can reinitiate their transcription.

The large-scale purification of native clock protein complexes from mouse liver throughout the day recently expanded our understanding of the size and complexity of these macromolecular complexes, as well as how they evolve in composition throughout the day [18]. Likewise, a genome-wide ChIP-Seq study mapping the temporal basis for DNA binding by the core circadian factors largely validated the canonical TTFL model of the vertebrate clock [92] (recently reviewed in [8]). However, this study also provided compelling evidence for the presence of two distinct repressive complexes on E-boxes: an early phase of repression characterized by association of PER1, PER2, CK1δ, CRY1, and CRY2 with CLOCK:BMAL1 on DNA, and a late phase where CRY1 alone was bound to CLOCK:BMAL1 on DNA (Figure 5) [92]. Collectively, these studies map out a new, more detailed depiction of the molecular basis for the vertebrate TTFL; the structural and biochemical details by which vertebrate core clock proteins assemble into these macromolecular complexes will be discussed below.

## Structural basis for transcriptional activation by CLOCK:BMAL1

The bHLH-PAS (basic helix-loop-helix PAS domain) family of transcription factors is characterized by a structurally ordered N-terminus with a bHLH DNA-binding domain followed by tandem PAS (PER-ARNT-SIM) domains and an intrinsically disordered C-terminus of variable length that contain transcriptional activation domains (TADs) required for the activation of gene expression. PAS domains are central to function of the bHLH-PAS family, as these modular domains provide specificity for heterodimeric partners within the family [93] and also interact with transcriptional co-regulators [94–96]. The crystal structure of the bHLH and tandem PAS domains of CLOCK:BMAL1 was the first to provide a detailed picture of the interdomain interactions that specify heterodimer formation in the bHLH-PAS family [97]. Subsequent structural studies of related bHLH-PAS complexes, including the hypoxia-inducible factor (HIF) [98], neuronal PAS (NPAS) [99] and aryl hydrocarbon receptor (AHR) [100] that all heterodimerize with ARNT (aryl hydrocarbon nuclear translocator), show similar interdomain orientations but have some differences in the overall structural organization that likely underlies their ability to bind distinct DNA motifs and interact with different transcriptional co-regulators [101]. In CLOCK:BMAL1, mutations at heterodimer interfaces in the bHLH domains and each of the tandem PAS domains weaken heterodimerization and result in reduced transcriptional activation in cells [97]. Furthermore, PAS domains could play a role in CLOCK:BMAL1 activation, as sumoylation of BMAL1 at K259 in the PAS-A domain targets CLOCK:BMAL1 to nuclear foci [102, 103].

Aligning the bHLH-PAS crystal structure with one of the CLOCK:BMAL1 bHLH domain bound to its cognate E-box [104] provides a model for the structural basis for DNA binding by CLOCK:BMAL1 (Figure 6A), although this model lacks any information about the disordered C-termini of CLOCK and BMAL1. Intrinsic disorder is common in

transcriptional factors, which are referred to as ‘malleable machines’ because they must engage with many different proteins to activate, repress or otherwise modulate their activity [105]. Indeed, nearly all of the proteins that coactivate or repress gene expression by CLOCK:BMAL1 bind to discrete regions within the disordered C-termini (Figure 6A), demonstrating their essential roles within the molecular circadian clock.

The transcriptional activation domain (TAD) at the very C-terminus of BMAL1 is essential CLOCK:BMAL1 function, as its deletion essentially eliminates transcriptional activation in cellular studies [106] and disrupts circadian rhythms *in vivo* [107]. The TAD interacts directly with the KIX domain of the pleiotropic transcriptional coactivators CBP and p300 [21, 25], which facilitate gene expression through rhythmic histone acetylation when they are recruited to DNA-bound CLOCK:BMAL1 in the morning [92, 108]. However, other regions in the BMAL1 C-terminus also contribute to regulation of CLOCK:BMAL1 activity. Using a chimera-based strategy, Liu and colleagues identified two regions in the disordered C-terminus that differentiate BMAL1 from its related paralog BMAL2, which cannot support sustained circadian oscillations at the cellular level [109]; substitution of the upstream ‘G’ region and the C-terminal TAD from BMAL2 into BMAL1 eliminates its ability to reconstitute cellular circadian rhythms [25]. Acetylation of BMAL1 by TIP60 at K537 in the ‘G’ region is essential for transcriptional elongation via recruitment of BRD4 and pTEFb [110, 111]. Future studies to determine the molecular basis by which these regions cooperate with post-translational modifications in BMAL1 to sustain circadian rhythms should illuminate important steps in the molecular circadian clock and help to identify how BMAL2 contributes to circadian rhythms [109].

The disordered CLOCK C-terminus constitutes nearly half of the protein (~470 out of 855 total residues) and includes an acetyl Co-A-binding motif [112] and a short helical region that is essential for circadian rhythms encoded by Exon 19 [113]. Deletion of Exon 19 leads to a dominant negative mutant that lengthens period and reduces the amplitude of cycling in heterozygotes or disrupts circadian rhythms altogether in homozygotes [114]. How does Exon 19 contribute to the assembly of complexes that activate CLOCK:BMAL1? Exon 19 is required to recruit the histone methyltransferase MLL1 (mixed lineage leukemia 1) to CLOCK:BMAL1 on chromatin each morning [115], although it’s not clear if this is mediated by a direct interaction of MLL1 with Exon 19 or by a change in the molecular architecture of the CLOCK:BMAL1 complex that is dependent on Exon 19 (Figure 6A). This region has also been implicated in binding to PER proteins, although also likely not through a direct interaction [116]. The 51 amino acids encoded by Exon 19 make an extended coiled-coil dimer that interacts directly with the repressor CIPC (clock-interacting protein circadian) to influence the phosphorylation state and activity of CLOCK:BMAL1 [117, 118]. Notably, the stoichiometry of the CLOCK Exon 19:CIPC complex consists of a coiled-coil dimer of CLOCK bound to a helix from CIPC (Figure 6B) [117]. The presence of Exon 19 is also required for the inhibition of CLOCK:BMAL1 by the CLOCK paralog PASD1, a cancer/testis antigen that uses an Exon 19-like sequence to suppress clock cycling in cancer cells [119]. This suggests that Exon 19 could dimerize CLOCK:BMAL1 complexes to create a macromolecular assembly that is then recognized by regulatory proteins for activation or repression.

One framework in which to consider oligomers of the CLOCK:BMAL1 heterodimer is through cooperative interactions at tandem E-boxes in the promoters of its top transcriptional targets. These sites are characterized by an upstream E-box (E1) that typically conforms to a consensus E-box sequence, and an imperfect E-box downstream (E2) that is unable to recruit CLOCK:BMAL1 on its own [120, 121]. Binding of CLOCK:BMAL1 to the first site is needed for recruitment to the second, and the precise spacing of these tandem sites (6–7 nucleotides, but no more or less) required for cooperative binding and circadian oscillations suggests that it may be dictated by structural restraints [120, 121]. A beautiful study by Takahashi and colleagues demonstrated that Exon 19 is required for cooperative binding of CLOCK:BMAL1 heterodimers to tandem E-boxes [122], suggesting that Exon 19 might play a critical role in the assembly of larger macromolecular assemblies on DNA (Figure 6C) that lead to subsequent recruitment of MLL1 [115] and other factors, like the PER proteins, that appear to require Exon 19 for recruitment, although perhaps not through a direct interaction [116]. Tissue-specific clock modifiers like the bHLH transcription factor MyoD (myoblast determination protein 1) also appear to capitalize on cooperative interactions with CLOCK:BMAL1 to regulate circadian rhythms in muscle [123]. We still have much more to learn about the structural basis of CLOCK:BMAL1 association with E-boxes and/or nucleosomes in their role as a pioneer-like transcription factor [124] and how tissue-specific enhancers modulate CLOCK:BMAL1 occupancy and activity on the genome [125].

## Tuning in and turning off—cytoplasmic assembly of CRY-PER-CK1 complexes

CLOCK:BMAL1 directs the expression of its own repressors as part of the central loop of the vertebrate TTFL [8]. These proteins initially assemble into at least two large complexes in the cytoplasm containing PER1, PER2 (and/or PER3), CRY1, CRY2, and the dedicated clock kinase, Casein Kinase 1δ (CK1δ) before they enter the nucleus to regulate CLOCK:BMAL1 activity (Figure 7A) [18]. Quantitative mass spectrometry analysis of these complexes suggests that changes in participation of the PER proteins and an accessory trafficking factor differentiate the cytoplasmic complexes, but a clear accounting for the stoichiometry of core clock proteins in either of these complexes has not yet been done [18]. Of the three PER proteins, only PER1 and PER2 have essential roles in the vertebrate TTFL [126] where they serve as stoichiometrically limiting factors in the assembly of clock protein complexes [127]. In line with this, constitutive overexpression of either PER1 or PER2 disrupts circadian rhythms [128], perhaps by titrating away key components and disrupting formation of CRY-PER-CK1 complexes.

What interactions determine assembly of the cytoplasmic CRY-PER-CK1δ complexes? They are likely scaffolded on the PER1/2 proteins, which share a conserved domain architecture with two tandem PAS domains in the N-terminus followed by a Casein Kinase 1-binding domain (CKBD) and a CRY-binding domain (CBD) in the C-terminus (Figure 7B). The PAS domain core of PER proteins facilitates their homodimerization (and likely heterodimerization [129]) through the second of two PAS domains, PAS-B (Figure 7C) [130, 131]. This PER-PER interaction is likely important for clock function, since an in-frame deletion of the core PAS-B motif in the *Per2*$^{Brdm}$ mutant leads to loss of circadian rhythms [132]. Analysis of the smaller cytoplasmic complex by mass spectrometry and western

blotting confirms that it contains the core repressor proteins PER1/2, CRY1/2, CK1δ, while blue native-PAGE suggests that the mass of this complex is ~900 kDa [18]. Based on what's known about the 1:1 stoichiometry of PER-CK1δ and PER-CRY interactions described below, the size of this complex suggests a tentative stoichiometry by mass of two PER dimers with each PER monomer stably bound to one molecule each of CK1δ and CRY. The stoichiometry of CRY1 and CRY2 may be different in these initial cytoplasmic assemblies, as the expression of *Cry1* is delayed by several hours relative to *Per1*, *Per2,* and *Cry2* and, as a consequence, its protein levels of CRY1 peak later than the other core clock proteins [19, 133].

Studies tracking PER abundance and subcellular localization throughout the day show that it remains anchored to its kinase throughout a cycle of the TTFL [18, 19]. The stable association of a kinase with its substrate all day long is rather unusual, but this anchoring likely helps to compensate for the poor activity that CK1δ has for a key regulatory site that serves as a rate-determining step for the control of PER stability [134, 135]; anchoring of the homologous CK1 and clock substrate was recently demonstrated to be important for circadian rhythms in the TTFL of *Neurospora crassa* [136]. This is likely true in the vertebrate TTFL as well, since overexpression of the isolated CKBD disrupts circadian rhythms by titrating the kinase away from PER proteins and the repressive complex [137]. There are no structures yet of a PER-CK1δ complex to demonstrate how anchoring occurs, but it requires two helical motifs within the CKBD of PER [138] that are presumed to make a 1:1 interaction between PER and the kinase.

The designation of CK1δ as the core vertebrate clock kinase, rather than its highly related paralog, CK1ε, follows from genetic studies and the use of isoform-selective inhibitors [139–141]. CK1δ controls PER2 abundance through a phosphoswitch mechanism [20] that integrates phosphorylation and other post-translational modifications (e.g., O-GlcNAc [142]) within the CKBD to regulate phosphorylation of an upstream degron that recruits the E3 ubiquitin ligase β-TrCP1 [143, 144]. However, it may not be quite that simple, as recent studies demonstrate that tissue-specific mRNA methylation regulates alternate splicing of CK1δ into two isoforms (δ1 and 2) that differentially influence circadian timing [145]. Intriguingly, the δ2 isoform shares considerable homology with CK1ε in a C-terminal region that controls kinase activity within the CKBD of PER2 [134], suggesting that the interplay between the different isoforms of CK1δ, as well as CK1ε, may contribute to tissue-specific control of circadian timing. Understanding the structural basis for incorporation of specific kinase isoforms into clock protein assemblies would help to resolve some of these questions.

The two vertebrate cryptochromes possess a ~55 kDa PHR (photolyase homology region) domain with a fairly high degree of conservation, but diverge in the sequence and length of their intrinsically disordered 'tails' [146]. The CRY tails contribute to circadian period and amplitude in genetic reconstitution studies of cellular clocks, but the PHR domain alone is sufficient to generate circadian rhythms when expressed in *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ cells [147, 148]. The PHR domain is structurally characterized by two pockets that are evolutionarily conserved with the DNA repair enzyme photolyase and light-sensitive CRYs, an FAD-binding pocket on one side and a secondary pocket on the other side (Figure 7D) [149]. The molecular basis for the high affinity interaction ($K_D$ ~20 nM) between PER and CRY

is fairly well understood; both PER1 and PER2 possess a CRY-binding domain (CBD) in their C-termini [150, 151] that leads to stable association of PERs and CRYs throughout the repressive loop of the TTFL [18, 19]. Crystal structures of CRY1-PER2 and CRY2-PER2 complexes reveal how the PER2 CBD wraps around the PHR domain of both cryptochromes in a similar manner [22, 23], winding down from the secondary pocket to make extensive contacts with the C-terminal CC (coiled-coil) helix, the last structural element of the PHR domain before the disordered tails (Figure 7D) [152]. Coordination of a molecule of Zn by two cysteines each from PER and CRY at the C-terminal end of the CBD suggests that redox regulation of this interface could play a role in remodeling the structural basis of PER-CRY assemblies [22, 23].

A series of competitive interactions at the CRY PHR regulate its stability and assembly into circadian repressive complexes. CRY stability is regulated by the E3 ubiquitin ligase FBXL3 [153–155]; however, it was demonstrated long ago that binding to PER somehow stabilizes the CRY proteins [19, 156], perhaps by incorporating them into these macromolecular assemblies. A crystal structure of CRY2 bound to FBXL3 by Zheng and colleagues revealed the molecular basis for this observation, showing that FBXL3 competes for an overlapping binding site on the CRY CC helix that competes directly with PER2 binding (Figure 7E) [24]. Competition also exists between FBXL3 and a related member of the family, FBXL21, which differ in their subcellular localization and ubiquitination activity, to further regulate CRY stability [157, 158]. The CRY2-FBXL3 structure revealed that FBXL3 also docks a flexible C-terminal extension into the FAD pocket of CRY2 (Figure 7E). This interaction is critical for FBXL3 binding, as titration of FAD can displace FBXL3 *in vitro* [24] and decreases in intracellular FAD stabilize the CRYs *in vivo* [159]. The discovery of exogenous small molecule regulators, like KL001 [160], that target the FAD-binding pocket in the CRY PHR domain to displace FBXL3 and stabilize CRYs [161] demonstrates that chemical biology approaches to regulate CRY stability and complex formation can exert a powerful effect on circadian timing.

Altogether, we now appreciate that assembly of CRY-PER-CK1 complexes depends on multiple factors, from a series of high affinity binding sites for CK1 and CRY on PER1/2 to interactions with regulatory proteins that compete for mutually exclusive binding sites. However, we have yet to fully incorporate new insights about how post-translational modifications on CRY regulate its stability and activity within the vertebrate TTFL into the molecular framework of these macromolecular assemblies [162–165]. With a structure-based framework in mind, we may be able to identify how modification of specific sites on CRY or PER proteins influences their protein dynamics and/or interactions to alter complex formation and therefore, circadian rhythms.

## Assembly and activity of early nuclear repressive complexes

After cytoplasmic complexes of core clock repressors make their way into the nucleus in the mouse liver, they pick up a number of additional factors and form a stable association with CLOCK:BMAL1 to swell to an apparent size of ~2 MDa [18]. Although prior studies revealed how these additional factors can contribute to transcription termination or deposit chromatin modifications that disfavor transcriptional activation [91, 166–171], none of these

factors appear to be stoichiometrically associated with the large nuclear assemblies [18]. However, use of single gene knockouts to selectively remove one core clock component or another profoundly disrupts stability of the entire complex, suggesting that a tight network of multivalent interactions amongst the core clock proteins is needed to maintain the complex throughout its purification from mouse liver [18]. Biochemical studies to map discrete binding sites on clock proteins that recruit these 'accessory' factors to the nuclear repressive complexes would help to identify how they contribute to the function of this complex; it stands to reason that PER proteins likely act as large, flexible hubs for additional protein-protein interactions that contribute to the formation of these nuclear macromolecular assemblies. For now, the apparent heterogeneity of additional, non-core clock factors here represents a challenge for their future biochemical and structural characterization, as well as determining which components are essential for repression of CLOCK:BMAL1.

How might the core clock proteins of the early nuclear assembly repress CLOCK:BMAL1 activity? Biochemical studies have recently revealed how cryptochromes might bridge the circadian repressor assembly with CLOCK:BMAL1 by identifying direct interactions between the PHR domain of CRY1 and two distinct sites on CLOCK:BMAL1 that are essential for repression [25]. First, CRY1 binds a solvent-accessible loop from the CLOCK PAS-B domain into its secondary pocket (Figure 8A) [172] to stably dock CRY onto the structured PAS domain core of CLOCK:BMAL1 [97]. Intrinsic differences in the dynamics of the serine loop that is adjacent to the secondary pocket of CRY1 and CRY2 underlie their different affinities for the PAS domain core of CLOCK:BMAL1 [173], which influences their repressive function in cells to control circadian timing [174]. PER proteins play a critical role in this context by equalizing the affinity of CRY1 and CRY2 for the PAS domains of CLOCK:BMAL1 by remodeling their serine loops [173, 174]. Altogether, these data demonstrate that recruitment of CRYs to the PAS domain core of CLOCK:BMAL1 is a crucial, rate-determining step in circadian rhythms. Second, the CC helix of CRY1 interacts directly with the BMAL1 TAD at a site that overlaps with its CBP/p300 KIX domain-binding site (Figure 8A) [21, 25, 172, 175, 176]. Binding to CRY sequesters the TAD from coactivators in a mechanism of direct repression that is conserved with other transcriptional factors like p53 [177–179]. Sequestration appears to occur similarly by CRY1 and CRY2 (in the presence or absence of PER2), suggesting that this mechanism of direct repression is intrinsic to the cryptochromes [173].

In addition to the direct repression of CLOCK:BMAL1 activity by cryptochromes, a number of recent studies also suggest that the early repressive complex leads to displacement of CLOCK:BMAL1 from DNA. ChIP-Seq studies show a marked decrease in CLOCK:BMAL1 occupancy at core E-boxes (defined by the ~1440 genes where all of the core clock proteins are recruited) coincident with recruitment of the early repressive complex (Figure 4A) [92]. Although this phase of repression appears to initiate on chromatin, it may conclude with displacement of CLOCK:BMAL1 from DNA (Figure 8B), similar to what is observed in the *Drosophila* TTFL [180]. Exciting new data suggest that the PER-dependent recruitment of CK1δ, which can phosphorylate CLOCK in the purified nuclear complex and reduce its affinity for DNA *in vitro* could underlie this 'displacement-type' repression [18]. These findings are consistent with prior work from Sancar and colleagues demonstrating that while CRY can efficiently repress CLOCK:BMAL1 while

it is bound to DNA, triggering the acute nuclear entry of PER results in displacement of CLOCK:BMAL [181]. However, this PER-dependent displacement of CLOCK:BMAL1 obligately depends on the co-expression of cryptochromes [182]. As of yet, there are no compelling studies showing a direct interaction between PER proteins and CLOCK:BMAL1; therefore, it seems likely that cryptochromes are needed to bridge the interaction of PER complexes with CLOCK:BMAL1. CRYs could also help by stabilizing the macromolecular architecture of PER complexes necessary for the displacement of CLOCK:BMAL1 from DNA [18].

Altogether, these data provide a framework for the observation that constitutive overexpression of the isolated CBD of PER2 disrupts circadian rhythms [128] by interfering with PER-CRY interactions, suggesting that the timely assembly and activity of the CRY-PER-CK1 core of the nuclear repressive complexes appears to lie at the heart of circadian repression. The activity of additional factors, like epigenetic modifiers, in this massive nuclear repressive complex likely plays an important role in remodeling the chromatin environment around E-boxes to help define and extend the repressive phase by creating a chromatin state that disfavors gene expression [92, 183].

## A unique role for CRY1 in the late repressive complex

Although CRY1 participates in the early repressive complex with the other core clock repressors, its peak recruitment to DNA-bound CLOCK:BMAL1 in mouse liver occurs much later in the repressive cycle at Circadian Time (CT) 0–4 (Figure 4A) [92]. Consistent with this observation, CRY1 is predominantly found in the massive nuclear repressive complexes isolated from the liver throughout the early part of the evening, but a substantial amount of free CRY1 is present in the later part of the evening [18]. A number of studies support both PER-dependent and independent role for CRYs in circadian rhythms [181, 184, 185], and highlight a particularly important role for CRY1 late in the repressive phase [186]. ChIP-Seq studies demonstrate that this complex forms without the apparent inclusion of PER proteins when the occupancy of CLOCK:BMAL1 on E-boxes is beginning to rise again [92], suggesting that this is a fundamentally different repressive complex than that observed earlier in the evening (Figure 8B). Formation of this poised and repressed state of CLOCK:BMAL1 with CRY1 on DNA plays a critical role in defining the phase of gene expression [187] and likely contributes to period determination [188, 189] and the generation of sustained cellular rhythms [190].

Two factors contribute to the ability of CRY1 to serve as a PER-independent repressor in the late repression phase of circadian rhythms. First, the delayed timing of *Cry1* expression with respect to the other core clock repressors is critical [19], because studies to reconstitute the TTFL in *Cry1*$^{-/-}$;*Cry2*$^{-/-}$ cells [147, 148, 174], SCN [191] or animals [192] all require use of the minimal *Cry1* promoter that encodes its delayed expression [133] to recover circadian rhythms. Second, CRY1 has a dramatically higher affinity (20-fold) for the PAS domain core of CLOCK:BMAL1 compared to CRY2 [173], helping to explain its ability to maintain stable interactions with CLOCK and BMAL1 on DNA *in vitro* [193] and *in vivo* [92] in the absence of PER proteins [174]. While more studies are needed to fully understand the composition and activity of the different circadian repressive complexes, new insights into

the molecular basis for clock protein interactions are helping to define how macromolecular assemblies control circadian rhythms.

## Some unexpected commonalities in the cyanobacterial PTO and vertebrate TTFL?

This review has discussed the stepwise interactions that regulate the assembly, activity, and disassembly or turnover of macromolecular assemblies that contribute to circadian timekeeping in the cyanobacterial PTO and the vertebrate TTFL. Aside from the obvious difference that one clock mechanism can function independently of transcription and translation while the other obligately depends on it, there are a few similarities that appear to arise from the biochemical regulation of their macromolecular assemblies. For example, the reliance on enzymes with low, temperature-compensated activity in steps that dictate complex assembly or stability of key components seems to be a hallmark of circadian enzymes from the cyanobacterial PTO (e.g., KaiC CI ATPase activity) [64, 194] to the vertebrate TTFL (e.g., CK1δ activity on the PER2) [134, 195, 196]. As we learn more about the structural and biochemical basis for macromolecular assemblies of these clocks, the convergence of both competitive and cooperative interactions at key regulatory sites is striking. Many of the competitive interactions identified thus far revolve around proteins that play a particularly important role in repression, such as KaiB in the cyanobacterial PTO [29, 42] and CRYs in the vertebrate TTFL [24, 25]. Furthermore, studies that identify and experimentally probe sources of cooperativity have the potential to address fundamental questions about biological oscillators [197]. With these studies just taking root in the field, it's exciting to think about the major questions on the horizon and the mechanistic insights will come to light in the future.

## Remaining questions and future challenges

Several decades of studying circadian rhythms at the organism and cell-based level have laid out the genetic network for timekeeping by biological clocks [8, 198], so biochemical and structural studies are now playing an increasingly important role in establishing clock mechanisms that will help bridge basic research and the clinic to improve human health. While our mechanistic insight into any of the eukaryotic TTFLs falls short compared to the intricate biochemical network of the cyanobacterial PTO, the combination of top-down and bottom-up approaches has the potential to stimulate new ways of thinking about TTFLs and circadian rhythms. Considerable effort has been spent quantifying the abundance and subcellular localization of core clock proteins; while these are clearly important factors for oscillatory systems, recent studies have shown that the *quality* of some clock proteins may be more important than their *quantity* [164, 199]. But what dictates the 'quality' of a protein? In this context, quality seems like a proxy for the biochemical determinants of protein function that arise from post-translational modifications, interactions with regulatory proteins and/or changes in structure or structural dynamics. We still have a long way to go before we understand this level of detail for most clock proteins outside of the cyanobacterial PTO.

Aside from needing to understand more about the molecular architecture of clock protein complexes, we have much more to learn about the factors that regulate assembly and disassembly or deactivation of these complexes, whether it be through changes in subcellular

localization, protein turnover, and/or post-translational modifications. For example, while studying the cyanobacterial PTO *in vitro* offers incredible advantages for mechanistic studies, Golden and colleagues have shown that clock proteins undergo a concerted change in subcellular localization *in vivo* from diffuse and dispersed during the day to a highly concentrated hub at one pole of the cell at night [200]—this is certain to factor into regulation of macromolecular assemblies of the Kai proteins and their output proteins by changing the local concentrations of clock components. New approaches, like those that incorporate genome code expansion to rapidly and reversibly control the timing and abundance of clock genes [192], are starting to offer ways to better bridge the divide from structural biology and *in vitro* biochemistry to cellular and behavioral studies to provide a more comprehensive picture of circadian rhythms.

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## Abbreviations used:

| | |
|---|---|
| **AHR** | aryl hydrocarbon receptor |
| **ARNT** | aryl hydrocarbon receptor nuclear translocator |
| **bHLH-PAS** | basic helix-loop-helix PAS domain |
| **BMAL1** | brain and muscle ARNT-like |
| **CBD** | CRY-binding domain |
| **CC** | coiled-coil |
| **ChIP-Seq** | chromatin immunoprecipitation-sequencing |
| **CIPC** | CLOCK-interacting protein circadian |
| **CKBD** | Casein Kinase 1-binding domain |
| **CLOCK** | circadian locomotor output cycles kaput |
| **CRY** | cryptochrome |
| **CT** | circadian time |
| **EM** | electron microscopy |
| **FBXL** | F-box and leucine-rich repeat protein |
| **FP** | fluorescence polarization |
| **HIF** | hypoxia-inducible factor |

| | |
|---|---|
| **IDP** | intrinsically disordered protein |
| **MD** | molecular dynamics |
| **MLL1** | mixed lineage leukemia 1 |
| **MyoD** | myoblast determination protein 1, myogenic NMR, nuclear magnetic resonance |
| **NPAS** | neuronal PAS |
| **PAGE** | polyacrylamide gel electrophoresis |
| **PAS** | PER-ARNT-SIM |
| **PER** | period |
| **PHR** | photolyase homology region |
| **PsR** | pseudoreceiver |
| **PTO** | post-translational oscillator |
| **SAXS** | small-angle x-ray scattering |
| **SCN** | suprachiasmatic nucleus |
| **TAD** | transcriptional activation domain |
| **TTFL** | transcription/translation feedback loop |

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**Highlights:**

- Most clocks rely on transcriptional cycles, but some can occur post-translationally
- Some biochemical principles may be shared by all clocks
- Clocks rely on the remodeling of core clock protein complexes throughout the day
- Slow biochemical or enzymatic steps are crucial for timekeeping in all clock types
- Clocks depend on competition for mutually exclusive binding at key interfaces

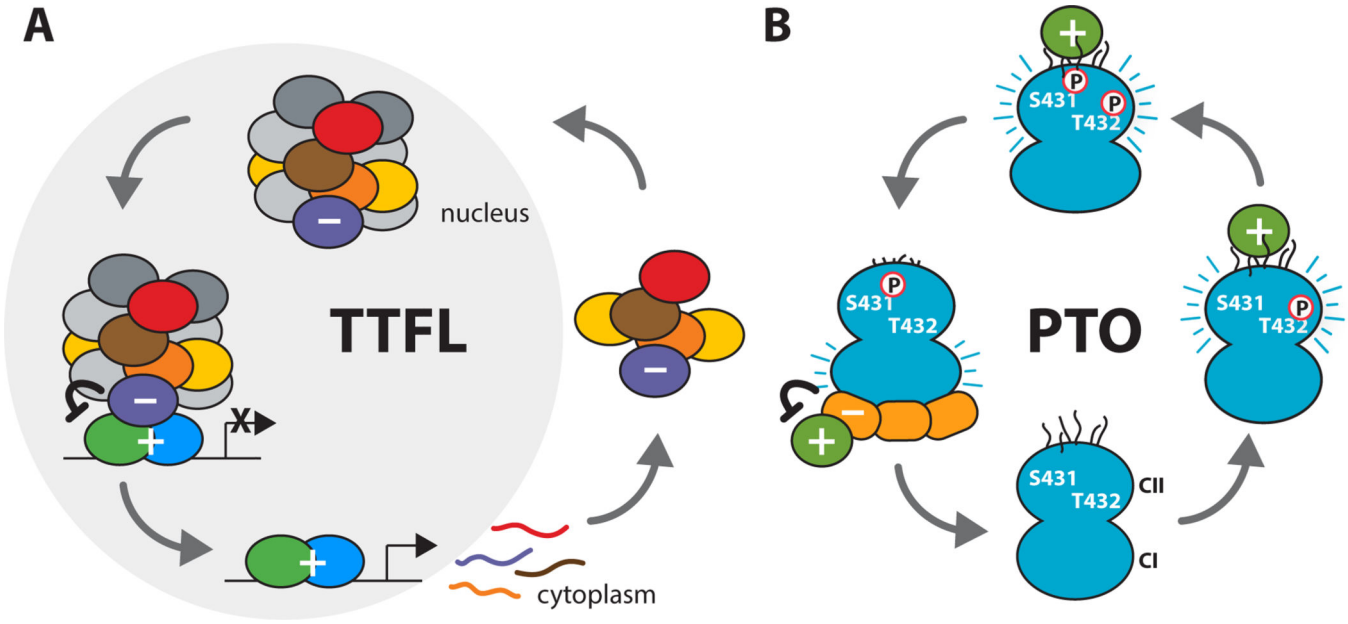


**Figure 1. Molecular architecture of two biological clocks**

**A**, A canonical transcription/translation feedback loop (TTFL) incorporates a transcription factor as the positive element (+), which drives the expression of its repressors that act as the negative element (–) in the feedback loop. Delays can be incorporated into the feedback loop at the level of transcriptional, post-transcriptional, translational, and/or post-translational regulation. Ultimately, the repressors inhibit the transcription factor until their abundance or activity decreases, and the TTFL begins again. **B**, A canonical post-translational oscillator (PTO) is based on rhythmic changes in protein activity and/or quaternary assemblies. In the cyanobacterial PTO pictured here, KaiC (cyan) begins the day in the unphosphorylated state. Autophosphorylation is initiated on T432, stimulated by KaiA (green) binding to C-terminal A-loop extensions, and then proceeds to S431. Retraction of the A-loops, ring-ring stacking of the CI and CII domains of KaiC, and ATP hydrolysis by the CI domain lead to KaiB binding (orange) and sequestration of KaiA in an inactive state. This allows for complete autophosphorylation of KaiC to reinitiate the PTO.

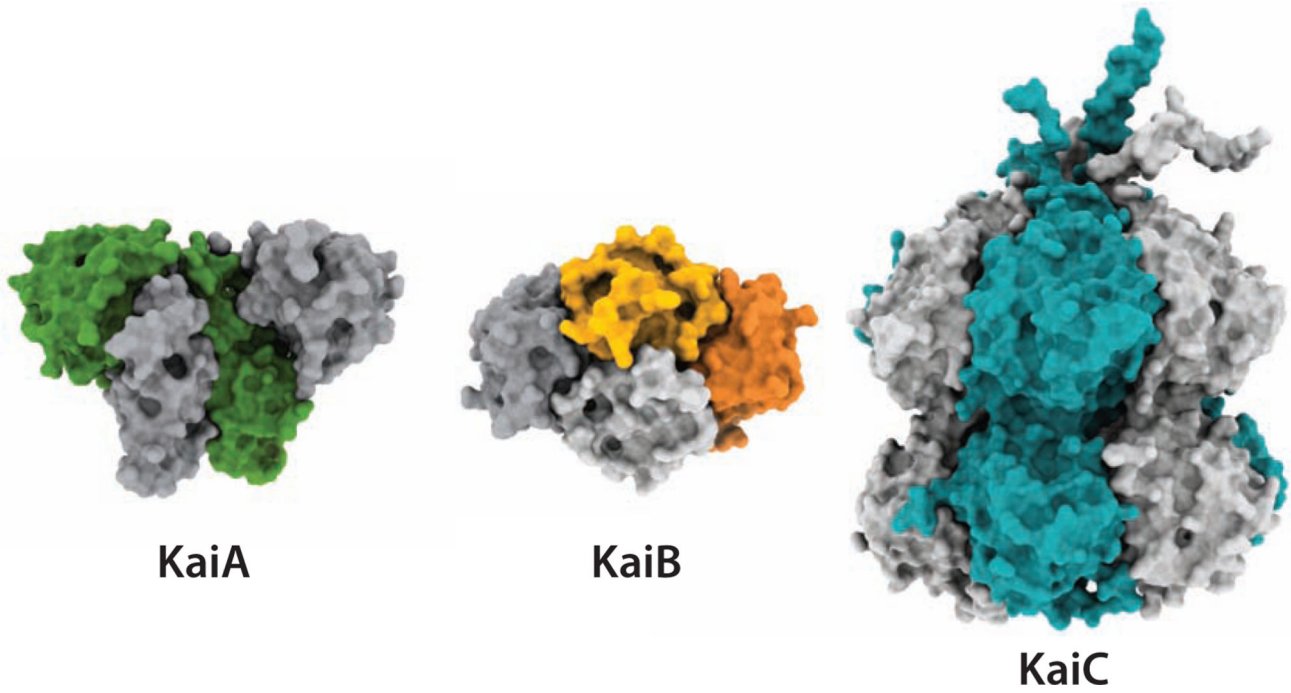


**Figure 2. The Kai proteins of the cyanobacterial PTO**

Crystallographic structures of the three Kai proteins from *Synechococcus elongatus* PCC 7942. The domain-swapped KaiA dimer is depicted with one subunit in green and one in dark gray (PDB: 1R8J). The KaiB tetramer (a dimer of dimers) is depicted with one core 'dimer' in orange and yellow with the other in dark and light gray (PDB: 4KSO). KaiC has tandem ATPase domains that organize into a hexamer with the N-terminal CI domain at the bottom and the C-terminal CII domain at the top with the C-terminal A-loop extensions depicted in this structure of the pSpT phosphostate of KaiC. Subunits of the hexamer are alternately colored in teal and light gray (PDB: 3DVL).

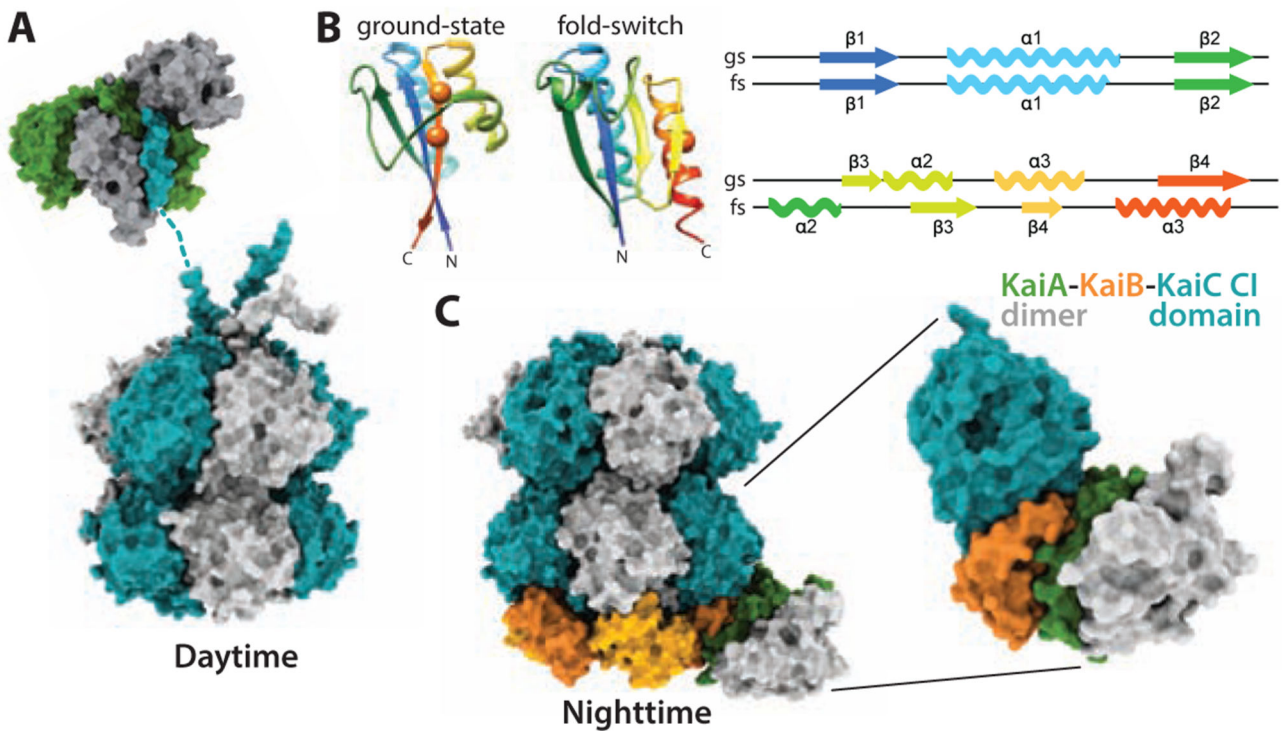


**Figure 3. Kai protein interactions that drive the cyanobacterial PTO**

**A**, During the daytime, the KaiA dimer (green/dark gray) binds a KaiC A-loop (teal) in the cleft between its C-terminal α-helical dimer interface (PDB: 5C5E). A flexible tether (dashed line) links the A-loop extension to the KaiC CII domain to stimulate nucleotide exchange. Subunits of the hexamer are alternately colored in teal and light gray (PDB: 3DVL). **B**, KaiB is a metamorphic protein. The structure of a monomer from the tetrameric ground state (PDB: 4KSO) is shown in rainbow coloring from the N-terminus (blue) to the C-terminus (red), with the Cα of two residues (G89 and D91) depicted as spheres. In the fold-switched monomer state (PDB: 5JYT), these two residues end up in a solvent-exposed C-terminal α-helix. The secondary structure notation of ground-state (gs) and fold-switch (fs) is shown at the right, color-coded to match the structural representations at left. **C**, The KaiABC complex assembles in the nighttime. A crystal structure of the KaiA ΔPsR domain dimer (green/light gray)-KaiB (orange)-KaiC CI domain (teal) subcomplex (at right, PDB: 5JWR) is modeled onto the crystal structure of a KaiBC hexamer with the S431E phosphomimetic mutation (PDB: 5JWQ). Subunits are alternately colored in KaiB (orange and yellow) and KaiC (teal and light gray) to highlight individual subunits.

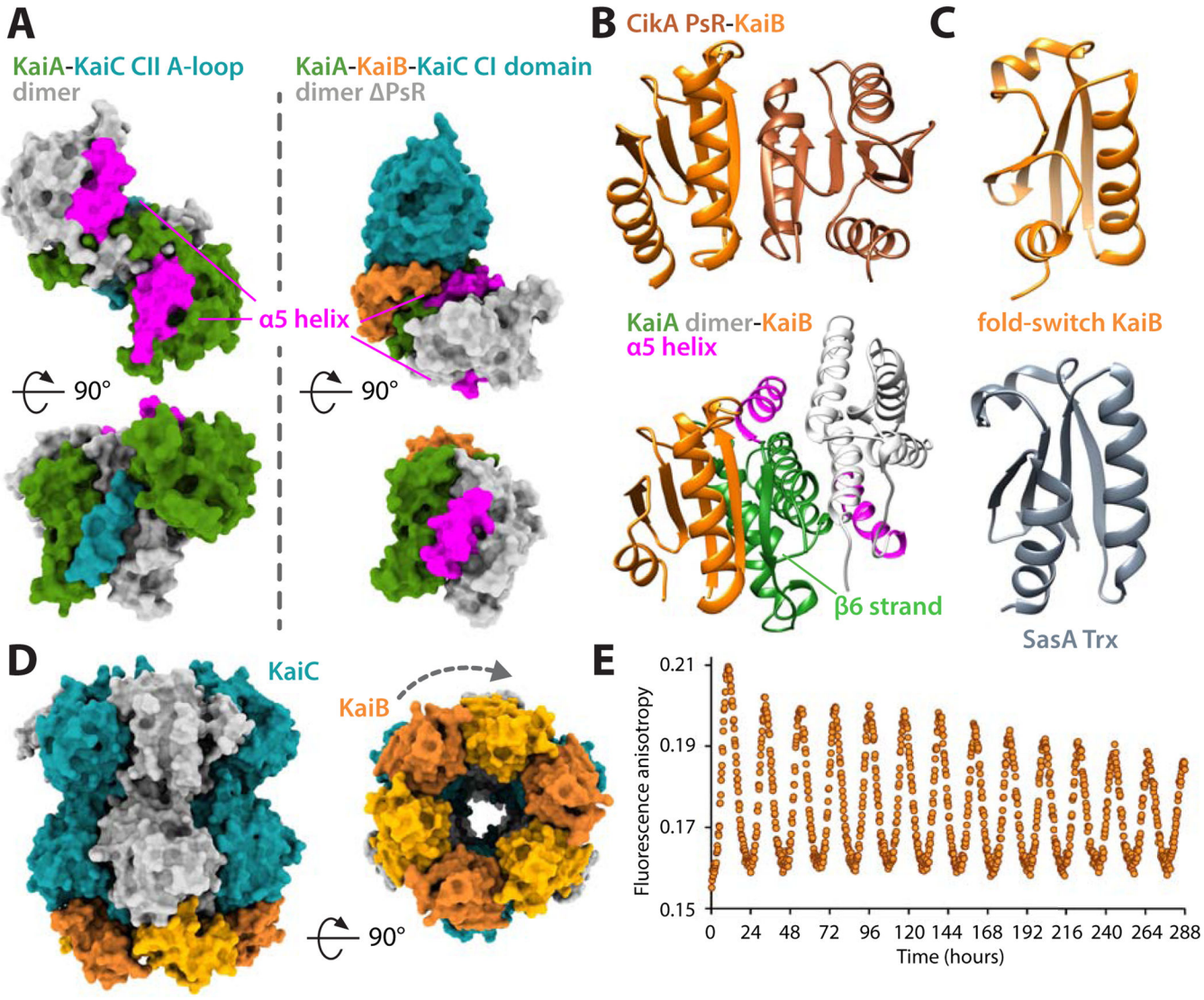


**Figure 4. Competition and cooperativity in the cyanobacterial PTO**

**A**, The α5 helix of KaiA (magenta) undergoes a major conformational change to form an autoinhibitory complex at night in the KaiABC complex. In the daytime, the α5 helix links the C-terminal dimer interface to the N-terminal PsR domains when KaiA is bound to the KaiC CII domain A-loop (left, PDB: 5C5E). At night, the α5 helix rotates out and down to occupy the same binding site as the KaiC CII A-loops (right, PDB: 5JWR). **B**, KaiA competes with the PsR domain of CikA for binding onto KaiB (in the context of the KaiBC complex at night). Top, the PsR domain of CikA (brown) forms an intermolecular β-sheet with the fold-switched conformation of KaiB (orange, PDB: 5JYV). Bottom, the same interface is used to recruit KaiA to the KaiBC complex via the β6 linker strand of one subunit of the KaiA dimer (green and light gray) to the fold-switched conformation of KaiB (orange, PDB: 5JWR). **C**, The thioredoxin-like fold of the fold-switched conformer of KaiB (orange, PDB:5JYT)) is highly similar to that of the N-terminal domain of SasA, which also binds to the KaiC CI domain (slate, PDB: 1T4Y). **D**, A crystal structure of the KaiBC hexamer with the S431E phosphomimetic mutation (PDB: 5JWQ). Subunits are alternately

colored in KaiB (orange and yellow) and KaiC (teal and light gray) to highlight individual subunits. The bottom view illustrates interactions between neighboring KaiB subunits that likely play a role in its cooperative recruitment to KaiC. E, A real-time fluorescence polarization-based assay for the cyanobacterial PTO monitors time-dependent association of fluorescently-labeled KaiB with KaiC and KaiA (image reprinted with permission from [86]).

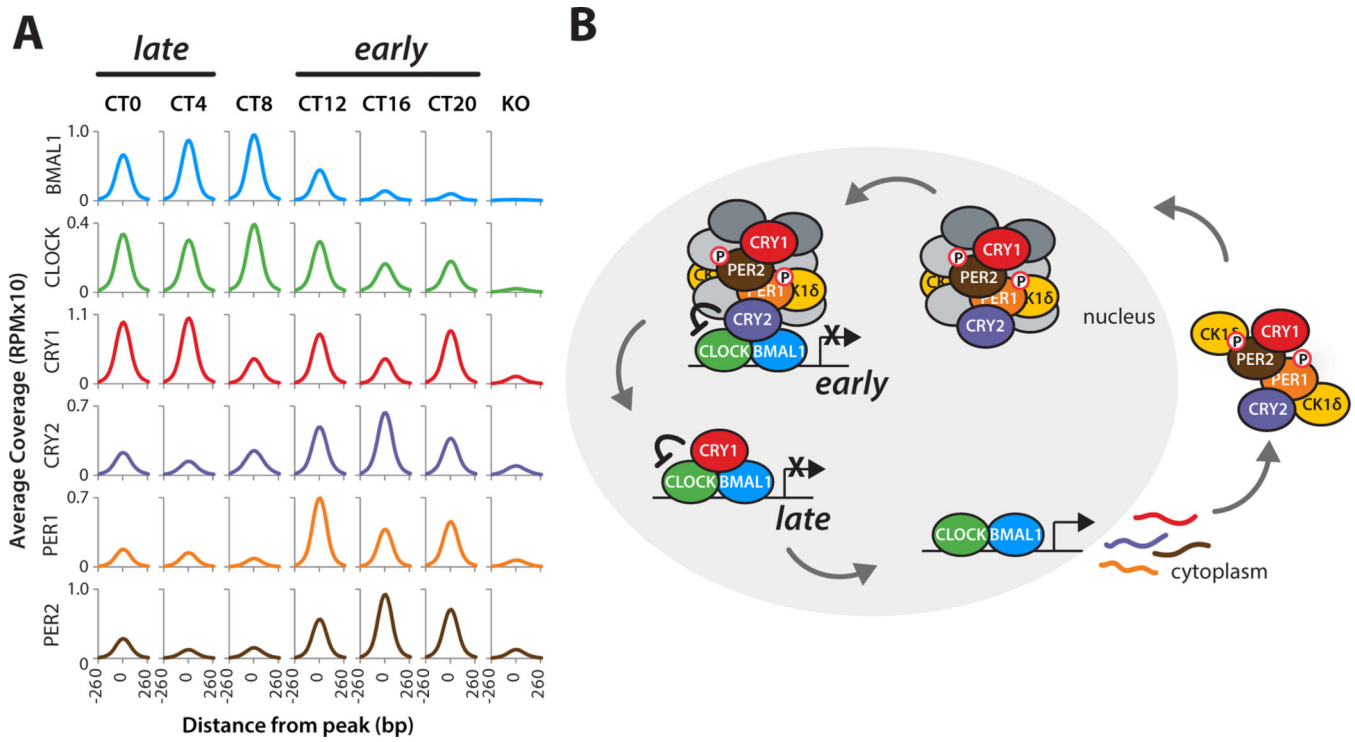


**Figure 5. Evidence for distinct DNA-bound repressive complexes in the vertebrate TTFL**

**A**, Circadian recruitment of core clock proteins to over 1400 share genomic binding sites in clock-controlled genes suggests formation of at least two distinct repressive complexes, an early complex defined by binding of BMAL1 (blue), CLOCK (green), CRY1 (red), CRY2 (purple), PER1 (orange) and PER2 (brown) from CT12-CT20, and a late complex defined by CRY1 binding to CLOCK:BMAL1 at CT0-CT4. Knockout (KO) mice were used as a negative control for each clock protein. Reprinted with permission from [92]. **B**, A cartoon schematic of the vertebrate TTFL depicting a model of the core clock proteins consistent with the ChIP-Seq data shown in panel A. The inclusion of extra, unnamed proteins (light and dark gray) in the nuclear repressive complexes is consistent with the observed increase in size from ~1 to 2 MDa in size [18].

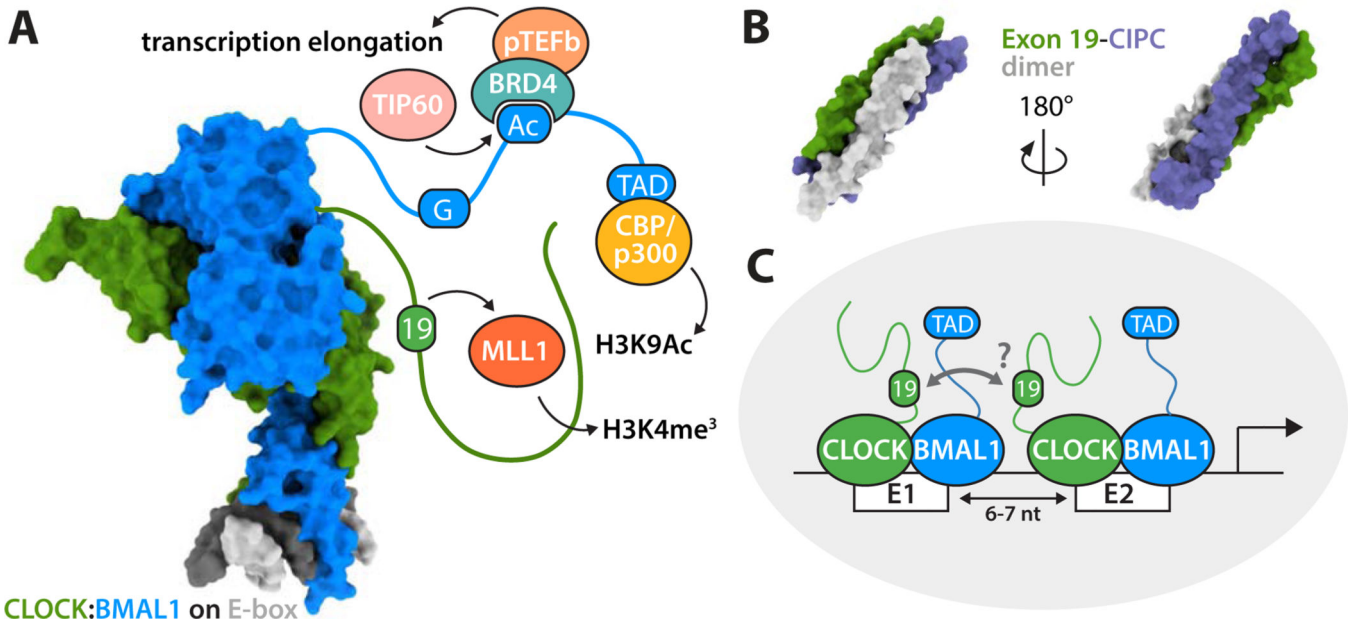


**Figure 6. Structural basis for CLOCK:BMAL1 activation and assembly on DNA**

**A**, A crystal structure of the CLOCK:BMAL1 bHLH-PAS heterodimer (PDB: 4F3L) overlaid with one of the E-box-bound bHLH domains (PDB: 4H10) illustrates how the bHLH and PAS domains of CLOCK (green) and BMAL1 (blue) contribute to heterodimer formation and DNA binding. Disordered C-terminal extensions on both CLOCK and BMAL1 include regions necessary for binding coactivators. In BMAL1, an acetylation site at K537 and the downstream TAD recruit distinct proteins needed for activation of gene expression. Likewise, the C-terminus of CLOCK possesses a 51-residue α-helical peptide encoded by Exon 19 that is required for recruitment of the histone methyltransferase MLL1. **B**, A crystal structure of the Exon 19 dimer (green and light gray) bound to the repressor CIPC (purple, PDB: 5VJX) suggests that regulation of Exon 19 dimerization and/or interactions could play a role in CLOCK:BMAL1 activity. **C**, A cartoon schematic illustrating the observation that the helical region encoded by Exon 19 is required for cooperative assembly of CLOCK:BMAL1 heterodimers onto tandem E-boxes.

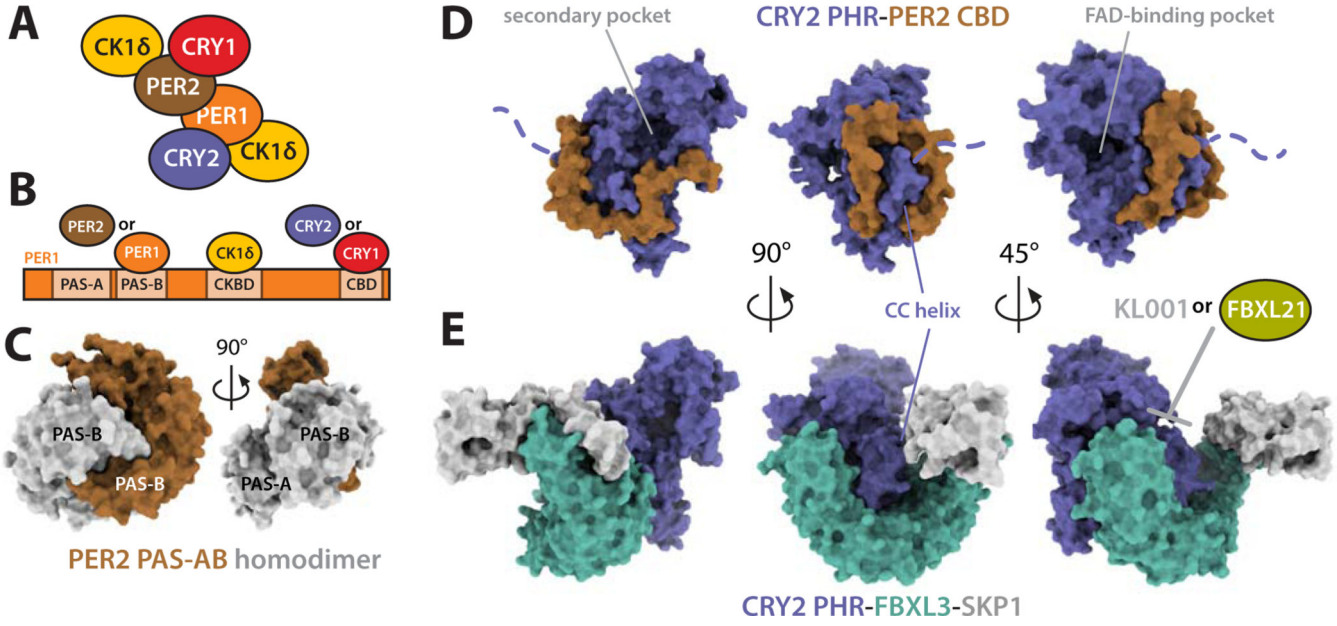


**Figure 7. PER proteins nucleate assembly of cytoplasmic repressive complexes**

**A**, A cartoon schematic of the core clock proteins found by mass spectrometry in minimal cytoplasmic assemblies purified from mouse liver. **B**, Domain schematic of PER1, illustrating domains that likely play a role in the assembly of core clock protein complexes, including homo- and heterodimerization by the PAS-B domain, the CK1δ-binding domain (CKBD), and the CRY-binding domain (CBD). **C**, A crystal structure of the PER2 PAS-AB homodimer with subunits colored in brown and light gray (PDB: 3GDI) demonstrates how PAS-B domains bind to one another to nucleate dimerization. **D**, A crystal structure of the CRY2 PHR domain (purple) with the PER2 CBD (brown, PDB: 4U8H)) demonstrates how the CBD makes extensive contacts the CC helix of the CRY2 PHR before the disordered C-terminal tail (dashed line). **E**, A crystal structure of the CRY2 PHR domain (purple) bound to the leucine-rich repeat domain of its E3 ubiquitin ligase FBXL3 (cyan) and the adapter Skp1 (light gray; PDB: 4I6J) reveals the molecular basis for competition between the PER2 CBD and FBXL3 for the CRY2 CC helix. FBXL3 binding to CRY2 is also competitive with another E3 ubiquitin ligase FBXL21 and the small molecule KL001, which targets the FAD-binding pocket to displace FBXL3 [160].

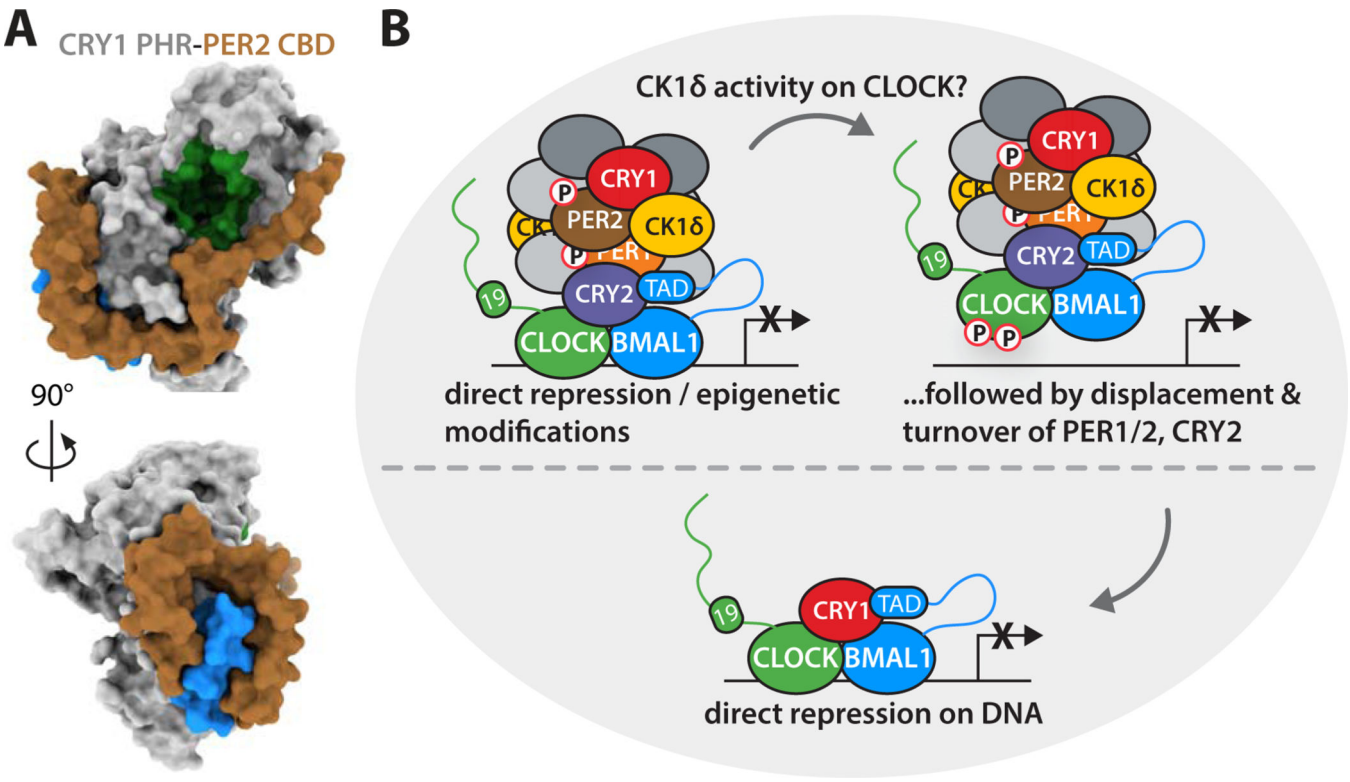


**Figure 8. Structural basis for repression of CLOCK:BMAL1 by CRY proteins**

**A**, A crystal structure of the CRY1 PHR domain (gray) bound to the PER2 CBD (brown, PDB: 6OF7) depicts how the PER2 CBD is tightly associated with the CLOCK-binding site in the CRY secondary pocket (green) and the BMAL1 TAD-binding site on the CC helix (blue). **B**, A cartoon schematic of early and late repressive complexes on DNA. Top, the formation of complexes with CLOCK:BMAL1 is likely nucleated by CRY proteins, which interact directly with CLOCK and BMAL1 and can inhibit their activity directly by sequestration of the BMAL1 TAD from activators; however, ChIP-Seq and biochemical data suggest that PER-containing complexes lead to displacement of CLOCK:BMAL1 from DNA in a CRY-dependent manner, possibly through phosphorylation of CLOCK by the dedicated clock kinase CK1δ. Bottom, CRY1 binds to CLOCK:BMAL1 on DNA to form the late repressive complex that maintains the transcription factor in a poised and repressed state until it is time to begin the TTFL again.