Chapter 12
The Kai-Protein Clock—Keeping Track of Cyanobacteria's Daily Life

Joost Snijder and Ilka Maria Axmann

Abstract Life has adapted to Earth's day-night cycle with the evolution of endogenous biological clocks. Whereas these circadian rhythms typically involve extensive transcription-translation feedback in higher organisms, cyanobacteria have a circadian clock, which functions primarily as a protein-based post-translational oscillator. Known as the Kai system, it consists of three proteins KaiA, KaiB, and KaiC. In this chapter, we provide a detailed structural overview of the Kai components and how they interact to produce circadian rhythms of global gene expression in cyanobacterial cells. We discuss how the circadian oscillation is coupled to gene expression, intertwined with transcription-translation feedback mechanisms, and entrained by input from the environment. We discuss the use of mathematical models and summarize insights into the cyanobacterial circadian clock from theoretical studies. The molecular details of the Kai system are well documented for the model cyanobacterium *Synechococcus elongatus*, but many less understood varieties of the Kai system exist across the highly diverse phylum of Cyanobacteria. Several species contain multiple *kai*-gene copies, while others like marine *Prochlorococcus* strains have a reduced *kaiBC*-only system, lacking *kaiA*. We highlight recent findings on the genomic distribution of *kai* genes in Bacteria and Archaea and finally discuss hypotheses on the evolution of the Kai system.

Keywords Circadian clock · Biochemical oscillator · KaiC · KaiB · KaiA · PTO · TTFL · Cyanobacteria · *Synechococcus elongatus* · *Prochlorococcus*

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Circadian Clocks

We have an intuitive notion of daytime and organize our daily lives accordingly, even without an exact definition or external measure of time. How do we do this? Our body, down to our individual cells, has inner clocks that enable all our rhythmic biological activities like sleeping, eating, body temperature, hormone levels … thinking.

Not only human life is adapted to the rotation of our planet Earth. Almost all living beings including animals, plants, and fungi possess an inner clock—a circadian clock that is defined by a 24-h period. The naming *circadian* originates from the Latin words *circa* meaning "around" and *dies* meaning "day". A fascinating feature of this biological clock is that it ticks very robustly despite daily changes in temperature, light, and nutrient availability. However, the inner clock can be entrained by exactly these cues, temperature, light, and nutrients. For example, when we travel across time zones, our inner clock stays robustly with the 'old daytime' and we experience jetlag. After a few days our body clock adapts to the new environmental light-dark cycle and jetlag is gone. This example also illustrates very clearly that our well-being is strongly affected by the synchrony of our body's clock with environmental cues. When there is a mismatch between our inner biological clock and the external environment, like in the case of jetlag, we don't feel well due to daytime fatigue and sleep disturbances. Moreover, chronic misalignment between our lifestyle and the rhythm dictated by our inner clock is associated with increased risk for various diseases ranging from mental disorders, cardiovascular and metabolic diseases, to cancer (Roenneberg and Merrow 2016).

Since the discovery of the genetic code and its information flow from DNA to RNA to proteins, the idea raised that there might exist a closing loop from the protein expressed in a cell back to its gene encoded on the DNA. Indeed, such a transcription-translation feedback loop, or TTFL, was found for the first-ever discovered circadian gene in fruit flies, named *period*, encoding the PERIOD protein. PER proteins prevent their own synthesis resulting in continuous, 24-h oscillations of PER protein levels in each single *Drosophil*a cell (Hardin et al. 1990; Vosshall et al. 1994). For their discoveries of molecular mechanisms controlling the circadian rhythm, Jeffrey C. Hall, Michael Rosbash, and Michael W. Young were jointly awarded with the 2017 Nobel Prize in Physiology or Medicine. This key mechanistic principle for the biological clock applies not only to humans but to all circadian clocks known so far.

How to test for a true circadian clock? In order to demonstrate an endogenous source for 24-h rhythmicity, the organism of interest is placed in a constant environment, for example constant darkness, excluding any influence of external stimuli. Now, behavior, physiology, gene expression, and any other relevant feature, which is expected to be under the influence of a free-running clock, can be monitored over time. Finally, if a 24 h free-running rhythm is found, it has to be robustly detected under altered external parameters. On the one hand, the rhythm should be resettable by exposure to external stimuli such as light and temperature, a process named

entrainment. The external stimulus that is able to entrain a rhythm is called the Zeitgeber. On the other hand, the 24-h rhythm has to maintain over a range of physiological temperatures despite the changing kinetics, a feature termed temperature compensation. In the case that all three criteria are fulfilled: endogenous free-running 24-h period, entrainment, and temperature-compensation, a true circadian clock exists.

Until the 1980s, circadian rhythms were believed to be restricted to eukaryotes, even though there was already strong evidence that cyanobacteria also schedule their physiological behavior into daily cycles (Wyatt and Silvey 1969; Gallon et al. 1974; Weare and Benemann 1974; Millineaux et al. 1981). Cyanobacteria are widespread in marine, freshwater and terrestrial environments, and many of them are capable of fixing atmospheric nitrogen making a major contribution to the global nitrogen cycle. Like Plants, Cyanobacteria depend on light to perform photosynthesis during the day, a process that provides the primary source of energy for almost all forms of life on Earth. Due to their capability of using light as the only energy source and CO_2 as feedstock, Cyanobacteria attract growing attention for the production of clean and sustainable energy and other valuable products. As a by-product of the photosynthetic light reaction Cyanobacteria produce oxygen, which interferes with certain biological processes like oxygen-sensitive nitrogen fixation. Cyanobacteria have solved this problem by separating the interfering processes in space and/or in time. For example, several filamentous cyanobacteria are able to differentiate specialized cells, named heterocysts that do not evolve oxygen and, thus, are able to fix molecular nitrogen. Unicellular Cyanobacteria, like those drawn in Fig. 12.1, usually schedule nitrogen fixation at night when oxygen is not being produced by photosynthesis. Here, it is important to note that the circadian model cyanobacterium *Synechococcus elongatus* sp. PCC 7942 (*Synechococcus elongatus*) is not able to fix dinitrogen. However, even under continuous illumination, a temporal separation of disparate processes persists providing the first strong evidence for an internal timing system—a circadian clock.

In the 1980s, several experiments cultivating cyanobacteria under constant light demonstrated 24-h rhythms of not only nitrogen fixation and photosynthesis, but also cell division at different temperatures (Grobbelaar et al. 1986; Stal and Krumbein 1987; Sweeney and Borgese 1989). Thus, cyanobacteria satisfy all criteria of a temperature-compensated circadian clock, although many species are unicellular and can divide faster than once a day (Johnson et al. 1996; Kondo et al. 1997). Today we know that nearly all metabolic activity in a cyanobacterial cell is orchestrated by a circadian clock to overcome simultaneous occurrence of incompatible processes. Obviously, even simple cyanobacteria have evolved an inner timing system to foresee the accompanying daily changes of light and temperature and regulate their physiology in daily cycles (Beck et al. 2014; Cerveny and Nedbal 2009; Diamond et al. 2015; Saha et al. 2016). Thus, the added efficiency provides an important benefit to life, and circadian clocks can be found in all domains of life.

The molecular mechanism underlying a circadian clock in Cyanobacteria remained elusive for another 20 years. First attempts to understand the molecular details of the cyanobacterial clock pointed to a cluster of three genes, *kaiA*, *kaiB* and

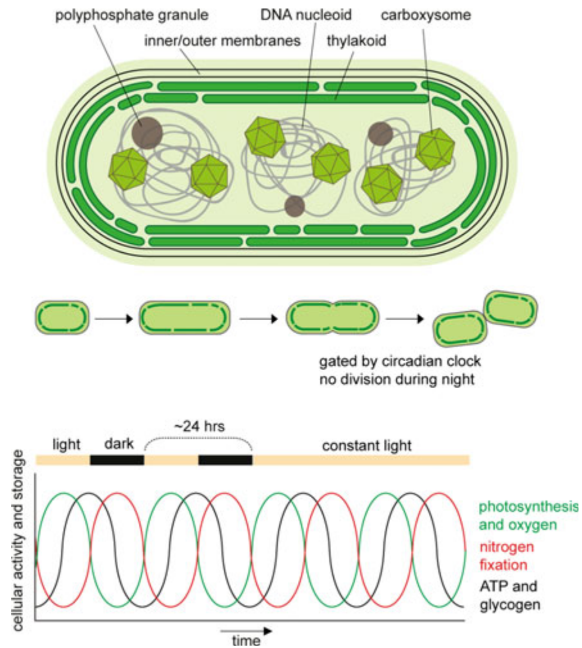

Fig. 12.1 A day in the life of a cyanobacterium. Schematic cellular structure of a single-celled cyanobacterium, like the genus *Synechococcus* (*top*). Schematic of cell division (*middle*). Physiological activities of a cyanobacterial cell under light-dark cycles, followed by constant light (*bottom*). Shown are photosynthesis, oxygen production, cellular ATP levels, glycogen storage, and nitrogen fixation. Please note that the circadian pattern of nitrogen fixation only applies to strains that fix dinitrogen from the environment. Circadian activities persist under constant light: a hallmark of circadian clocks

kaiC (Ishiura et al. 1998). In agreement with the well-established eukaryotic model, a TTFL was suggested for cyanobacteria. Negative feedback control of *kaiC* expression by KaiC itself was suggested to generate the circadian oscillation. Almost 10 years later, in 2005, the TTFL model for cyanobacteria was completely revised: firstly, it was demonstrated that circadian oscillations continue without transcription and translation in darkness (Tomita et al. 2005). Secondly, a very elegant in vitro experiment mixing the three purified recombinant Kai proteins, KaiA, KaiB, and KaiC, together with ATP unambiguously illustrated beautiful 24-h oscillations of KaiC phosphorylation over several days at different temperatures in a test tube (Nakajima et al. 2005). Thus, the underlying oscillatory mechanism in cyanobacteria relies on protein-phosphorylation and is evolutionary not related to transcriptional-translational based circadian systems prevailing in human, animals, plants, and other eukaryotes. Cyanobacteria's biochemical, post-translational oscillator is instead made of only three proteins: KaiA, KaiB, and KaiC, which share no sequence similarity to any known eukaryotic clock component and are sufficient to generate temperature-compensated circadian oscillations of protein phosphorylation in vivo and even in vitro (Nakajima et al. 2005).

In the following chapter, we provide insights into the Kai-protein system of *Synechococcus elongatus*—the best-studied circadian clock of Cyanobacteria, Sect. 2. We illustrate structural details of the three Kai proteins, KaiC, KaiB, and KaiA, Sect. 3. In Sect. 4, we explain how the time signal is transferred by protein factors from the protein clock to the DNA, orchestrating global patterns of gene expression, and how the clock receives input from the environment. The

cyanobacterial protein clock is intertwined with several feedback mechanisms that allow for robust 24-h behavior. However, the clock can be entrained by environmental signals. Mathematical models allow us to describe and understand this complex network, and we summarize insights from theoretical studies of the cyanobacterial system, Sect. 5. Although the unique three-protein oscillator is well documented for the model cyanobacterium *Synechococcus elongatus*, information is largely missing for the highly diverse phylum of Cyanobacteria. Several species contain multiple *kai*-gene copies, others like marine *Prochlorococcus* strains harbor only a reduced *kaiBC* system, thus, lacking the *kaiA* gene. We highlight recent findings on the genomic distribution of *kai* genes in Bacteria and Archaea. Finally, we discuss hypotheses on the evolution of the Kai-protein system, Sect. 6.

The Kai-Protein System of *Synechococcus Elongatus*

The circadian clock of cyanobacteria drives expression of most of the cell's gene products, including the clock genes themselves (Guerreiro et al. 2014; Ito et al. 2009; Markson et al. 2013; Xu et al. 2013). Dozens of those gene products are directly involved in sensing light, nutrients, or temperature and relay their input to get the biological clock more in tune with the environment. Transcription and translation feedback on the clock genes is therefore an important part of maintaining a robust circadian rhythm, but at its core the biological clock of cyanobacteria is a purely post-translational oscillator (Axmann et al. 2014; Hosokawa et al. 2013; Kitayama et al. 2008; Qin et al. 2010b; Teng et al. 2013; Tomita et al. 2005). It is known as the Kai system, derived from the Japanese *kai*, meaning 'cycle'.

The oscillator consists of just three gene products: KaiA, KaiB, and KaiC derived from the *kaiA* gene and a *kaiBC* operon (Ishiura et al. 1998). The three components of the Kai system go through a twenty-four-hour cycle of assembly-disassembly and post-translational modifications that determines the phase of the circadian rhythm (Kageyama et al. 2003; Kitayama et al. 2003; Nishiwaki et al. 2004; Xu et al. 2004). With only three components the Kai system is remarkably simple. So simple in fact, that it can be reconstituted in a test tube, just by mixing the purified Kai proteins in the presence of ATP and magnesium ions (Nakajima et al. 2005). Even outside the cell, in the test tube, the purified Kai proteins will run through their twenty-four-hour cycle of assembly and post-translational modifications (see Fig. 12.2). The cyclic reaction will persist for weeks, provided that there is enough ATP. This test-tube oscillator presents an amazing opportunity to study the molecular details of biological timekeeping in cyanobacteria and it has taught us much of what we know about the Kai system today.

KaiC is the central component of the oscillator. It is composed of two internally duplicated domains: KaiC-CI at the N-terminus and KaiC-CII at the C-terminus. Both domains bind ATP (Hayashi et al. 2003, 2004, 2006; Mori et al. 2002; Nishiwaki et al. 2000; Pattanayek et al. 2004). Binding of ATP is required for the domains to form hexamers: one ring of KaiC-CI and another of KaiC-CII. The rings

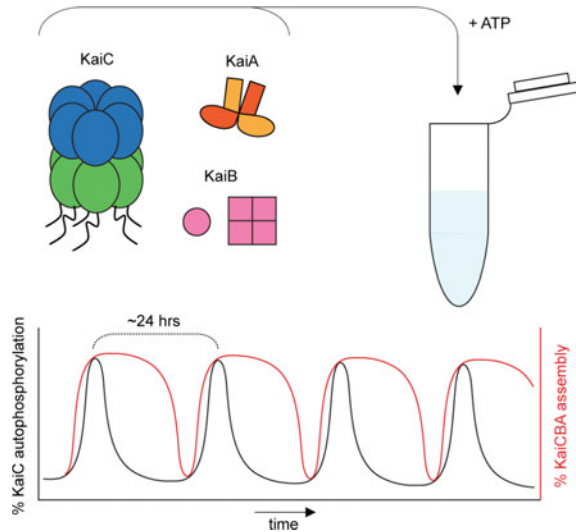

Fig. 12.2 A circadian clock in a test-tube. Kondo and colleagues demonstrated in 2005 that the KaiABC system can be reconstituted in vitro from the purified recombinant protein products. Mixing KaiC, KaiB, and KaiA in the presence of magnesium and ATP produces a temperature-compensated circadian oscillation of KaiC autophosphorylation and KaiCBA complex assembly. This circadian clock in a test-tube has since become an invaluable tool to researchers across the globe studying the molecular details of Kai

are stacked on top of each other and connected by flexible linkers between the two domains of each subunit. The KaiC-CII domain has autokinase and autophosphatase activity (Iwasaki et al. 2002; Nishiwaki et al. 2000, 2004; Pattanayek et al. 2004; Williams et al. 2002; Xu et al. 2004). It sequentially phosphorylates a Threonine (T432), then a Serine residue (S431) across the interface of the subunits, followed by sequential dephosphorylation of the Threonine and Serine residues in the same order (Hayashi et al. 2006; Kitayama et al. 2013; Nishiwaki et al. 2007; Rust et al. 2007). The resulting levels of autophosphorylation set the phase of the circadian oscillation.

The KaiC-CI domain has an ATPase activity that tracks the circadian rhythm of KaiC-CII autophosphorylation (Abe et al. 2015; Murakami et al. 2008; Terauchi et al. 2007). This is mediated by the stacking interactions between the two rings (Chang et al. 2011, 2012; Oyama et al. 2018). Whereas the KaiC-CI domain always forms a tight hexamer in the presence of ATP, the KaiC-CII domains are more loosely associated and interact strongest when S431 is phosphorylated. As a result, the KaiC-CII domains form a tighter hexamer during one-half of the phosphorylation cycle, during which the two rings can also stack together more efficiently to promote the ATPase activity in the KaiC-CI domains. Together, phosphorylation and ATPase activity of KaiC determine how the hexamer interacts with the KaiA and KaiB components. In turn, the interactions with KaiA and KaiB modulate both the autophosphorylation and ATPase activities of the KaiC hexamer. This feedback between the enzymatic activities of KaiC and the interactions with KaiA and KaiB ultimately drive the circadian oscillations of the system (see Fig. 12.3) (Brettschneider et al. 2010; Clodong et al. 2007; Iwasaki et al. 1999; Kageyama et al. 2003; Kitayama et al. 2003; Nishiwaki et al. 2004; Qin et al. 2010a; Rust et al. 2007; Murakami et al. 2016).

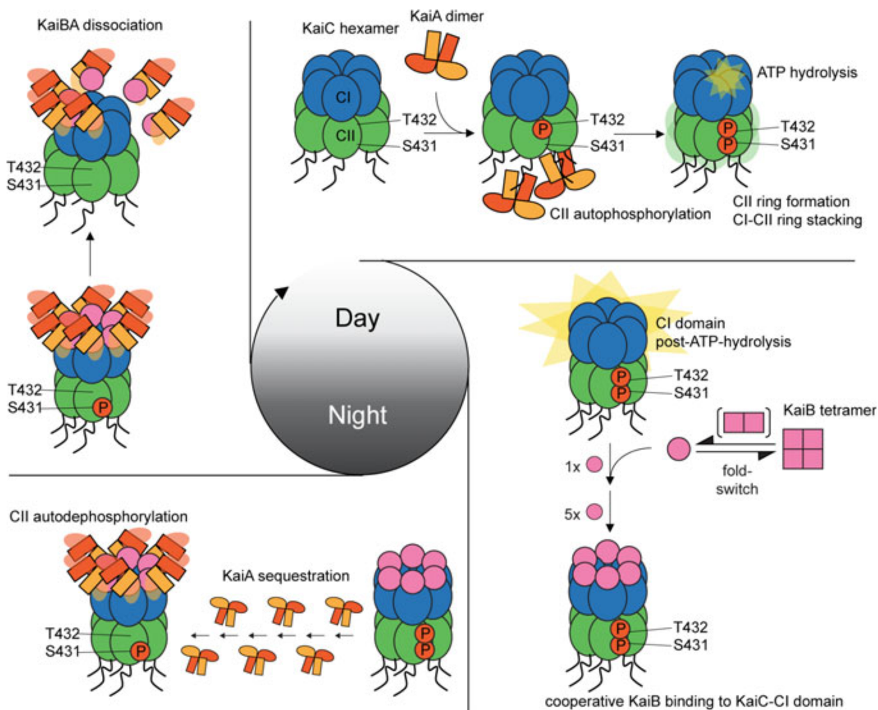

Fig. 12.3 Molecular mechanisms of the Kai system. During the subjective day, KaiA triggers sequential autophosphorylation of KaiC hexamers at T432, followed by S431 in the KaiC-CII domain. KaiC-CII autophosphorylation enhances CII ring formation and stacking interactions with the CI ring. These stacking interactions between the CII and CI rings couple ATPase activity in KaiC-CI to KaiC-CII autophosphorylation. The KaiC-CI domains in their post-hydrolysis conformation cooperatively bind six KaiB monomers in a fold-switched state. Fold-switched KaiB in the KaiCB complexes presents an alternative binding site for KaiA, sequestering the pool of free KaiA. While sequestered KaiA is no longer available to promote KaiC autophosphorylation, the KaiC hexamers revert to sequential autodephosphorylation of T432, followed by S431. Dephosphorylation of S431 releases KaiBA complexes from the KaiC hexamers and brings the circadian oscillation full circle

The autokinase and -phosphatase activities of KaiC are always at play simultaneously. Phosphatase activity of KaiC will dominate by default, resulting in progressively lower levels of autophosphorylation in the KaiC-CII domain. In the unphosphorylated state, KaiC can engage in an interaction with KaiA that promotes the autokinase activity of the KaiC-CII domain (Iwasaki et al. 2002; Williams et al. 2002). This results in sequential autophosphorylation of T432, followed by S431. Phosphorylation of S431 will promote the formation of the KaiC-CII ring to enhance stacking interactions with the KaiC-CI ring and elevate ATPase activity in this domain. In turn, ATP hydrolysis in the KaiC-CI domain triggers an interaction with KaiB (Chang et al. 2012; Mukaiyama et al. 2018; Mutoh et al. 2013;

Phong et al. 2013; Snijder et al. 2017; Tseng et al. 2017). When KaiB binds to KaiC, it exposes a new binding site for KaiA that outcompetes the autokinase-stimulating interaction with the KaiC-CII domain. By depleting the pool of KaiA, KaiB brings KaiC back to its default dephosphorylation activity, resulting in sequential dephosphorylation of T432, followed by S431. The interaction with KaiB will persist until S431 is finally also dephosphorylated. Dephosphorylation of S431 results in dissociation of KaiB and KaiA, bringing KaiC full circle to a free and unphosphorylated state that is capable of interacting with free KaiA again. In short, KaiA stimulates autophosphorylation of KaiC, which triggers a sequestration feedback mechanism that depletes the pool of free KaiA in KaiCB complexes to initiate a phase of dephosphorylation that brings the reaction full circle.

By stimulating the autokinase activity of unphosphorylated KaiC, KaiA drives the free-running circadian rhythm of autophosphorylation. KaiA is a two-domain protein that forms dimers through a domain-swapping interaction (Ye et al. 2004). It has a pseudoreceiver (PsR) domain on the N-terminus that is connected with a linker region to an alpha-helical C-terminal domain (Garces et al. 2004; Uzumaki et al. 2004; Vakonakis et al. 2004b; Williams et al. 2002). KaiA has two modes of interaction with KaiC, depending on the phase of the autophosphorylation cycle. The swapped C-terminal domains of KaiA form a cleft in the dimer that binds to the extended C-terminus of the KaiC-CII domain (Pattanayek and Egli 2015; Vakonakis et al. 2004b). This mode of binding is available to KaiA at all phases of the circadian rhythm and is required for the autokinase-stimulating interaction with KaiC. During most of the cycle, a KaiC hexamer can bind a single KaiA dimer at the C-termini of the KaiC-CII domains, except during the phosphorylation phase, when a second KaiA dimer can bind, promoting the autokinase activity of KaiC-CII (Brettschneider et al. 2010; Hayashi et al. 2004; Snijder et al. 2017). While S431 is phosphorylated and the two KaiC rings stack together, promoting ATP hydrolysis in the KaiC-CI domain and KaiB binding, a second mode of binding becomes available to KaiA. During this dephosphorylation phase of the cycle, KaiB exposes a new binding site that asymmetrically binds one subunit of a KaiA dimer at the linker region between the PsR- and the C-terminal alpha-helical domains (Snijder et al. 2017; Tseng et al. 2017). This mode of binding outcompetes the autokinase-stimulating interaction at the KaiC-CII termini, giving way to the dominant phosphatase activity of KaiC-CII again.

The interaction of KaiB with KaiC marks the dephosphorylation phase of the circadian oscillator. KaiB is a single-domain protein, in equilibrium between a monomeric and tetrameric form (Snijder et al. 2014). It has the remarkable ability to switch between two folded states (Chang et al. 2015). In the tetramer it adopts a unique KaiB fold, whereas it switches to a thioredoxin-like fold when it is bound to KaiC (Garces et al. 2004; Hitomi et al. 2005; Iwase et al. 2005; Pattanayek et al. 2008; Snijder et al. 2014; Villarreal et al. 2013; Tseng et al. 2017). Six KaiB monomers bind cooperatively to the six KaiC-CI domains of the hexamer (Lin et al. 2014; Snijder et al. 2014). The KaiB tetramer is therefore thought to act as a sink that dampens fluctuations in KaiB concentration. KaiB interacts with the KaiC-CI domains in their post-hydrolysis conformation, which is how the interaction is

triggered by ATPase activity of KaiC. Fold-switched KaiB bound to KaiC-CI exposes a new binding site for KaiA dimers, away from the KaiC-CII domain where KaiA would be able to stimulate autokinase activity. The KaiB-KaiA complex remains bound for as long as S431 is phosphorylated. When S431 is finally also dephosphorylated, the KaiB-KaiA complex is released from the KaiC hexamer and subsequently dissociates into the individual KaiB and KaiA components.

It is important to note that the Kai system is not a single-molecule timing mechanism. It is rather a biochemical network that produces circadian oscillations based on the ensemble behavior of the tens-of-thousands of copies of the Kai components that are present inside a single cyanobacterial cell. We have described an important succession of events on the molecular level, but it is unlikely that the signal from a single KaiC hexamer going through those steps will rise above the noise to produce robust circadian rhythms of gene expression. A typical cell will carry on the order of 10^4 copies of KaiB and KaiC, together with 10^3 copies of KaiA (Chew et al. 2018; Kitayama et al. 2003). This large excess of KaiBC over KaiA will ensure efficient KaiA sequestration during the dephosphorylation phase of the cycle.

The system has also evolved a mechanism to ensure synchronicity between the vast numbers of copies of the Kai components. During the dephosphorylation phase of the cycle, KaiC hexamers will exchange subunits to result in more homogeneous levels of autophosphorylation in the ensemble (Brettschneider et al. 2010; Clodong et al. 2007; Ito et al. 2007; Lin et al. 2014; Mori et al. 2007). This is especially relevant in the context of translation feedback on the circadian clock. The monomer shuffling will ensure that newly translated KaiC subunits mix with the existing pool of Kai components, thereby factoring into the phase of autophosphorylation in the entire ensemble of KaiC hexamers. Furthermore, all three Kai components also exhibit circadian patterns of subcellular localization (Cohen et al. 2014; Kitayama et al. 2003). Based on the workings of the test-tube oscillator such localization patterns are not essential for circadian oscillations per se but might be required in the context of other cellular processes like photosynthesis, nitrogen fixation, and cell division.

Our description of the Kai system so far corresponds to the biological clock as it is observed in *Synechococcus elongatus*, which has historically been an important model system for cyanobacterial research. We will discuss the evolutionary origins and diversity of Kai systems in more detail later, but would like to highlight one important variation on the Kai system here. Whereas in *Synechococcus elongatus* cells, KaiA is responsible for driving a free-running circadian rhythm of autophosphorylation in KaiC, there are other cyanobacteria that lack the KaiA component altogether (Schmelling et al. 2017). These include the ecologically widespread and important genus of *Prochlorococcus*. In these Kai systems that lack a KaiA component, the biological timer works via a fundamentally different mechanism. Rather than generating free-running rhythms of KaiC autophosphorylation with KaiA, which are rather robust to environmental stimuli and continue

oscillating even in constant dark or light conditions, the KaiBC-only systems work more like an hourglass, which has to be reset every day.

In the KaiBC-only systems, environmental stimuli like light or temperature will set off a period of KaiC autophosphorylation, followed by a period of KaiB binding and a drop in the levels of autophosphorylation (Chew et al. 2018). These two periods together last for approximately twenty-four hours. The KaiBC timer will set off a twenty-four-hour global gene expression pattern, enough to anticipate a new dawn, but will require the stimuli of a new day to persist in a circadian rhythm. The KaiBC systems are more in tune with the environment, but therefore simultaneously less robust in this regard. They are also less sensitive to relative noise that arises from the overall expression levels of the clock component, compared to KaiA-based systems, which require higher expression levels to maintain a robust twenty-four-hour cycle (Chew et al. 2018). It is thought that the trade-off between KaiBC and KaiBC-KaiA systems lies in their relative robustness to environmental stimuli and the costs of producing higher levels of KaiBC and KaiA. The KaiBC-only systems are also more common in genera that live in open-ocean surface waters in tropical and subtropical regions, whereas the KaiA-based systems are more common in genera that live in temperate climates that experience larger seasonal variations.

Structures of the Kai Proteins

KaiC

The two-domain structure of KaiC is the result of an internal gene duplication event (Ishiura et al. 1998). Even though the KaiC-CI and -CII domains have diverged substantially on a functional level, they remain remarkably similar on a structural level (Pattanayek et al. 2004). The similarity of the domains relates to their common ATP binding activity, with conserved Walker A motifs (also known as P-loops) and downstream Walker B motifs present in both KaiC-CI and KaiC-CII domains, responsible for the ATPase and autokinase/-phosphatase activities (Nishiwaki et al. 2000). In addition to their shared structural motifs, the domains each contain characteristic regions, and they are connected with a conserved flexible linker (see Fig. 12.4a) (Schmelling et al. 2017). The KaiC-CI domain contains a B-loop that changes conformation upon ATP hydrolysis and is a crucial component of the KaiB binding site (Abe et al. 2015). Compared to KaiC-CI, the KaiC-CII domain has an extended C-terminus containing two additional functional motifs. These mediate the interaction with KaiA and its autokinase-stimulating activity. It consists of a so-called ‘A-loop’, followed by the KaiA-binding region at the very end of the C-terminus.

The ATP-binding pockets in KaiC are formed at the subunit interface of the assembled hexamer (see Fig. 12.4b–d) (Abe et al. 2015; Egli et al. 2012, 2013;

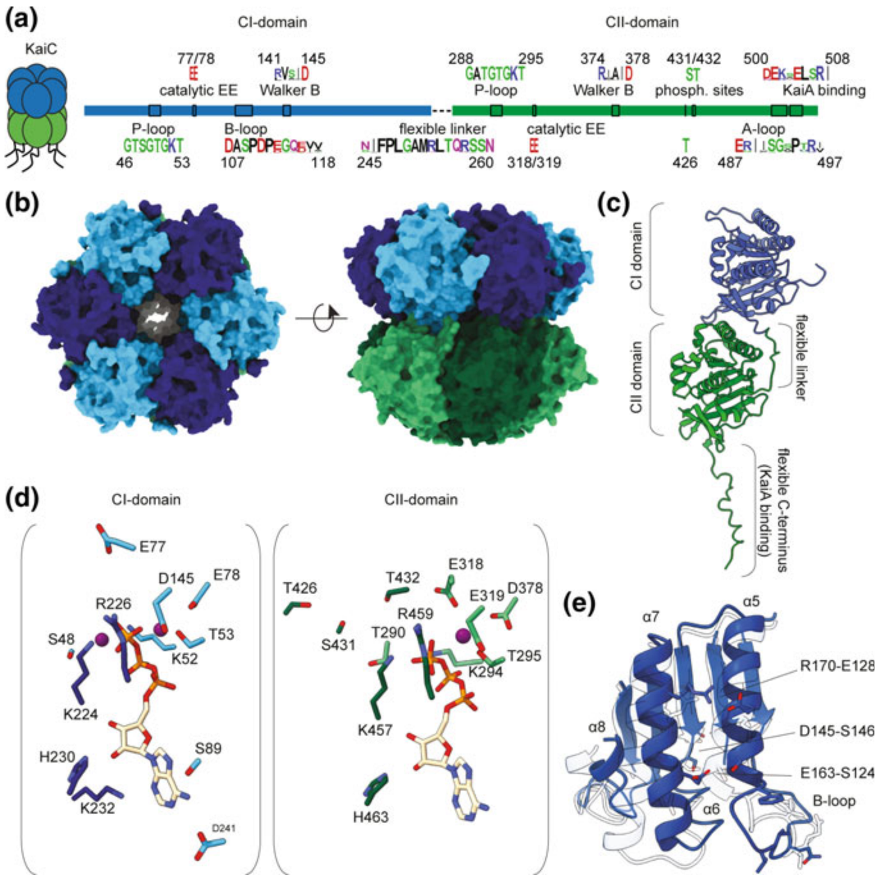

Fig. 12.4 Structure of the central clock component KaiC. **a** Domain structure and conserved sequence motifs in *Synechococcus elongatus* KaiC homologs. Sequence logos with residue numbers are shown for important functional motifs. **b** Surface representation of KaiC hexamers (PDB ID 1TF7). KaiC is shown along the central channel of the CI-domain hexamer (*left*) and in a 90-degree rotation showing stacking of the CI and CII domains (*right*). The flexible C-termini of the CII domain are not displayed. **c** Ribbon diagram of a KaiC subunit, including the flexible C-terminus of the CII domain (PDB ID 3DVL). **d** Detailed view of ATP binding in the CI and CII domains. Light and dark colors represent side chains from two different neighboring subunits. Magnesium ions are shown as purple spheres. Based on PDB ID 1TF7 and 4O0 M. **e** The KaiC-CI domain in the post-hydrolysis conformation (solid blue, PDB ID 4TLA) overlaid with the pre-hydrolysis conformation (transparent, PDB ID 1TF7)

Pattanayek et al. 2004, 2006, 2009, 2011, 2014; Xu et al. 2004). The ATPase and autokinase/-phosphatase activities of KaiC are thereby coupled to oligomerization. The triphosphate groups of the ATP molecules are fixed in position by coordinating interactions with two catalytic magnesium ions on either side of the neighboring subunits. The subunits themselves coordinate the magnesium ions through

interactions with P-loop residues (S48/K52/T53 in KaiC-CI, T290/K294/T295 in KaiC-CII) and three additional acidic residues (E77/E78/D145 in KaiC-CI, E318/E319/D378 in KaiC-CII). Residues E77 and E318 in the KaiC-CI and KaiC-CII domains, respectively, are crucial catalytic sites for ATP hydrolysis. Additional hydrogen bonds directly between the gamma-phosphate and two basic residues of the neighboring subunit (K224/R226 in KaiC-CI, K457/R459 in KaiC-CII) further stabilize the triphosphate group of ATP. The 2′-hydroxyl group of the ribose moiety is contacted by H230 in KaiC-CI, H463 in KaiC-CII. In the KaiC-CI domain, additional residues (S89/K232/D241) interact with the nucleobase portion of the ATP's through hydrogen bonds, but similar stabilizing interactions are missing in the KaiC-CII domain.

In the KaiC-CII domain, the gamma-phosphates of the ATP molecules are positioned within hydrogen-bonding distance of the phosphorylation sites that define the phase of the circadian rhythm. Note that most of the stabilizing interactions with ATP and in particular the interactions with the catalytic residues come from a neighboring subunit with respect to the phosphorylation sites. The circadian rhythm is defined by sequential autophosphorylation of T432 first, then S431, and followed by autodephosphorylation in the same order. A third adjacent residue, T426, can be transiently phosphorylated and is crucial to facilitate the circadian oscillation of autophosphorylation at T432/S431 (Pattanayek et al. 2009; Xu et al. 2004, 2009). It forms hydrogen bonds with phosphorylated S431 and shuttles the phosphate group during dephosphorylation, which happens through an ATP synthase and subsequent ATPase mechanism (Egli et al. 2012; Nishiwaki and Kondo 2012). Autophosphorylation of T432 results in additional intersubunit contacts between the transferred phosphate group and R385, as well as the catalytic E318 side chain. Phosphorylation of S431 results in new hydrogen-bonding interactions with H429 of the same subunit, which is in turn hydrogen bonded to D427 of the adjacent subunit. The autophosphorylation reaction thus changes the subunit interface, providing a structural basis for its effect on KaiC-CII hexamerization and the stacking interactions between the KaiC-CI and KaiC-CII rings.

Whereas the autophosphorylation cycle in the KaiC-CII domain results in no noticeable conformational changes beyond side chain rearrangements, ATP hydrolysis in the KaiC-CI domain results in allosteric conformational changes through long stretches of the polypeptide backbone (see Fig. 12.4e) (Abe et al. 2015). One of the three acidic residues that coordinate the triphosphate group of ATP, D145, undergoes a cis-to-trans isomerization with S146 in the ADP bound state. This changes the conformation of alpha helices α6 and α7 that lie directly beyond the D145-S146 peptide. Helix α6 further connects to α5, which follows the conformational changes upon ATP hydrolysis. Helix α5 forms the KaiB-binding site together with the directly preceding B-loop. Helices α6 and α5 run in antiparallel direction on the outer face of the KaiC-CI domain. They connect through Van der Waals interactions as well as hydrogen bonds between E163-S124 and a salt bridge between R170-E128. The movement of α5 changes the conformation of the preceding B-loop, resulting in large side chain rearrangements for Q115, V117, V118, and F121. This is how ATP hydrolysis in the KaiC-CI domain

allosterically triggers KaiB-binding. Helix α6 further connects to α8, which also changes backbone conformation after ATP hydrolysis. It is positioned on the opposite subunit interface compared to the D145-S146 peptide and in close proximity to the neighboring ATP triphosphate. The functional role of α8 has not yet been explored experimentally, but it likely also contributes to the allosteric changes upon ATP hydrolysis that trigger the binding of KaiB.

KaiB

KaiB has the astonishing ability to switch between two different folded states: a unique KaiB fold and a thioredoxin-like fold (see Fig. 12.5a, b) (Chang et al. 2015; Garces et al. 2004; Hitomi et al. 2005; Iwase et al. 2005; Pattanayek et al. 2008; Snijder et al. 2017; Tseng et al. 2017; Villarreal et al. 2013). The unique KaiB-fold is found in the tetrameric form of KaiB, whereas the thioredoxin-like fold is found in the KaiC-bound monomeric form. It is not clear whether KaiB switches fold as a free monomer, or whether KaiC-binding induces the transition. The two folds share a common strand-helix-strand motif in the N-terminal half of the polypeptide chain, with both β-strands contributing to the same central β-sheet. In the unique KaiB-fold, the second β-strand leads into a long loop running across the central β-sheet, followed by a very short strand-helix motif, the long α3 helix running antiparallel to the α1 helix, leading into the β4 strand on the C-terminus that completes the central β-sheet. In the thioredoxin-like fold, the common strand-helix-strand motif is followed by the α2 helix running across the central β-sheet, followed by two short antiparallel β-strands that complete the central β-sheet and lead into a long α3 helix on the C-terminus, running parallel to the α1 helix. The role of the unique KaiB-fold and tetramer is currently not well understood, but it has been suggested that the KaiB-tetramer can act as a sink to produce a more stable free KaiB-monomer concentration over a wider range of KaiB expression levels (Nakajima et al. 2010; Snijder et al. 2014).

Six KaiB monomers with the thioredoxin-like fold bind cooperatively to the KaiC-CI domain in the post-hydrolysis conformation (Snijder et al. 2017; Tseng et al. 2017). The newly formed α2 helix of the thioredoxin-like fold is an important part of the KaiC-KaiB interface, sitting inside the B-loop of KaiC-CI and directly contacting the α5 helix that changes conformation in the post-hydrolysis state (see Fig. 12.5c). There are direct contacts between α1 of KaiB and α3 of the neighboring KaiB subunit. These contacts could provide a potential mechanism for the cooperative binding, though these interactions have yet to be explored experimentally on a functional level. KaiC-CI intersubunit interactions may just as well contribute to the cooperative binding observed for KaiB, and the relative contribution of these factors remains to be determined.

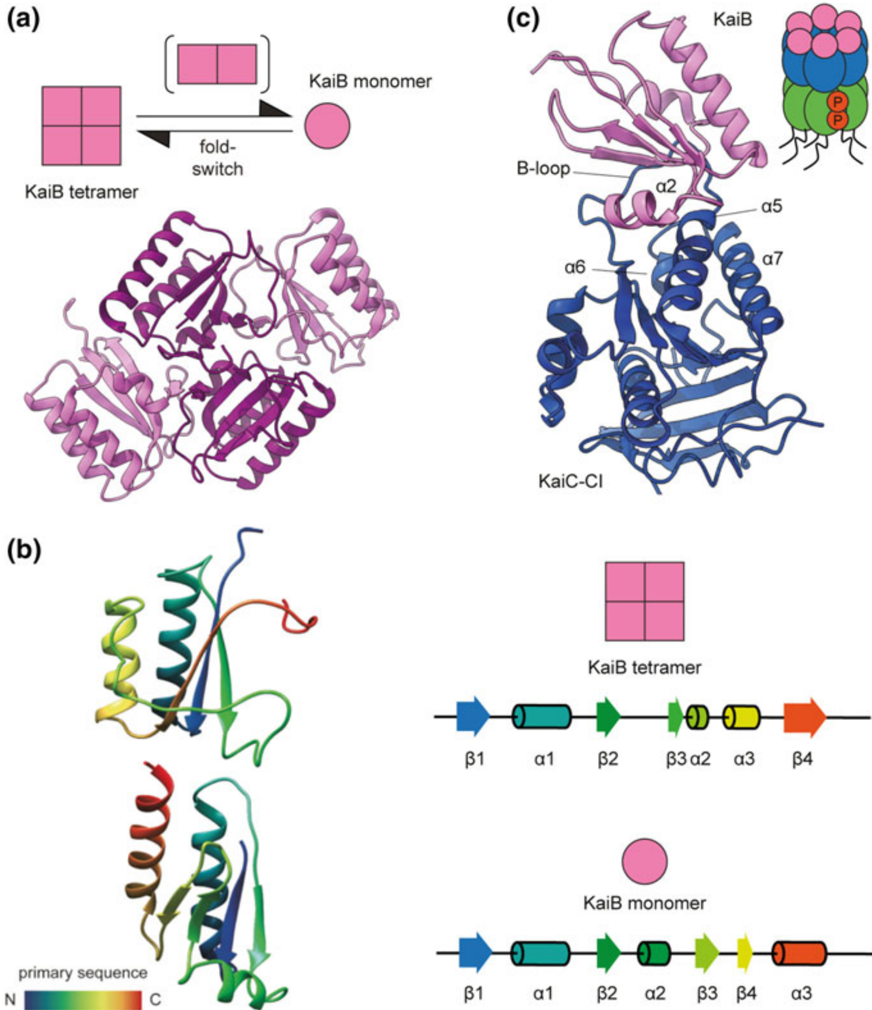

Fig. 12.5 Structure of KaiB and interactions with KaiC. **a** Ribbon diagram of the KaiB tetramer (PDB ID 4KSO). **b** Comparison of KaiB folds of the tetramer (top) and thioredoxin-like monomer (bottom). Ribbon diagrams are colored in rainbow gradients from N- to C-terminus, with corresponding diagrams of their secondary structures on the right. **c** Fold-switched KaiB in complex with the KaiC-CI domain (PDB ID 5JWO)

KaiA

KaiA is a two-domain protein that forms homodimers through a domain-swapping interaction (see Fig. 12.6a) (Ye et al. 2004). At the N-terminus is a Pseudo-Receiver domain, followed by a linker region and an alpha-helical domain on the C-terminus. The C-terminal alpha-helical domains are packed alongside each

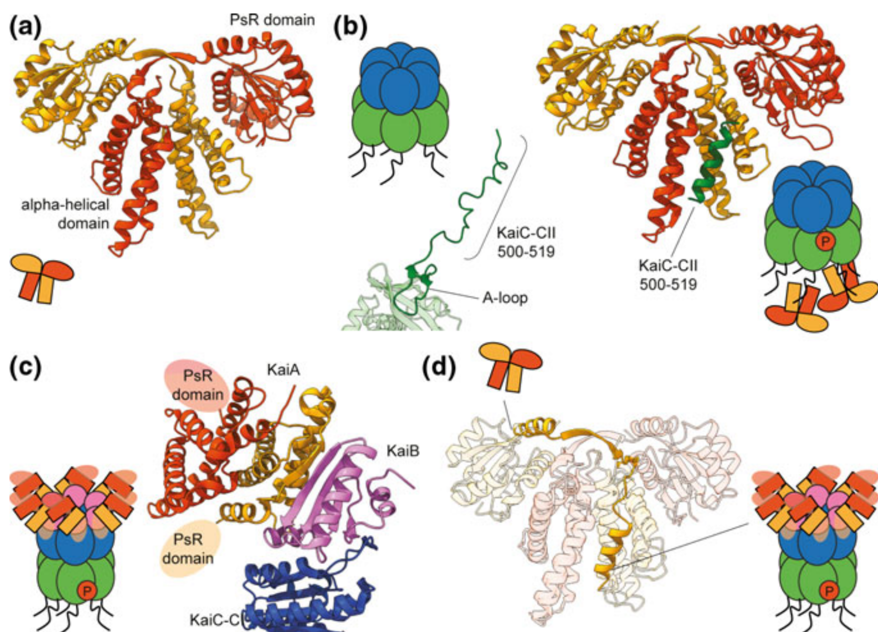

Fig. 12.6 Structure of KaiA and interactions with KaiC and KaiB. **a** Ribbon diagram of the KaiA dimer (PDB ID 1R8J). **b** Detailed view of the KaiC A-loop and KaiA-binding C-terminus (PDB ID 3DVL). Ribbon diagram of KaiA dimer in complex with a peptide derived from the KaiC C-terminus (PDB ID 5C5E). **c** Ribbon diagram of KaiA dimer in complex with KaiCB (PDB ID 5JWR). The KaiA dimer binds asymmetrically with a single subunit to KaiB. KaiCB binding displaces the PsR domains of KaiA, which are no longer visible in reconstructions of the KaiCBA complexes. **d** Detailed view of the conformational change of the PsR domain in KaiA upon KaiCB binding (PDB ID 1R8J and 5JWR). The linker region between the PsR domain and alpha-helical domain swings out in front of the KaiC-binding cleft of the KaiA dimer

other in a V-shape, forming an open cleft at one end of the dimer. On the opposite end, where the two helical bundles meet at the hinge of the V-shape, the subunits crossover with the linkers to the PsR domain running in antiparallel direction. The linkers consist of short β-strands, together forming a small antiparallel β-sheet that leads into the PsR domains on either side.

The cleft formed by the alpha-helical domains of the KaiA-dimer constitutes the KaiC-binding region (see Fig. 12.6b) (Pattanayek and Egli 2015; Vakonakis et al. 2004b). The long flexible C-terminus of the KaiC-CII domain contains the corresponding KaiA-binding region at residues 499–513, which is directly preceded by the so-called A-loop at residues 487–497. In the absence of KaiA-binding, the A-loops dip into the central channel of the KaiC-CII ring. When the A-loop is deleted, it results in constitutive hyperphosphorylation of KaiC (Kim et al. 2008). This effect is mediated through putative interactions of the A-loop with P-loop residues and a loop directly preceding the phosphorylation sites T432/S431/T426 (Egli et al. 2013). It has been suggested that KaiA-binding to the KaiC-CII domain

results in extension of the A-loop, such that it is threaded out from the central channel of the KaiC-CII domain. It supposedly activates KaiC autophosphorylation activity in a similar manner to deletion of the A-loop. Consistent with this model, the A-loop sequence has diverged in KaiBC-only systems like those found in *Prochlorococcus* (Axmann et al. 2009; Schmelling et al. 2017). Though these observations clearly point to an important role for the A-loop, the molecular mechanism by which KaiA binding activates KaiC autophosphorylation is not well understood at present.

Autophosphorylation of KaiC triggers a sequestration feedback on KaiA through the phosphorylation-dependent ATPase activity of the KaiC-CI domain, which triggers KaiB binding (Brettschneider et al. 2010; Snijder et al. 2017; Tseng et al. 2017). KaiB in the thioredoxin-like fold bound to KaiC-CI domain presents an alternative binding site to KaiA that outcompetes the interaction on the termini of the KaiC-CII domains. KaiB interacts with KaiA primarily through contacts with the β2 strand of the central β-sheet (see Fig. 12.6c). It binds asymmetrically to a single subunit of the KaiA dimer, contacting the β-strand linker that connects the PsR and alpha-helical domains. The β-strand linker and connected PsR domains of KaiA undergo a large displacement that facilitates binding to the β2 strand of KaiB (see Fig. 12.6d). Rather than crossing over at the hinge of the V-shape formed by the alpha-helical domains, the β-strand linker, and PsR domain now swing over in front of the central cleft of the KaiA dimer that binds the KaiC-CII termini. It has been proposed that this conformation of KaiA represents an autoinhibited state since the β-strand linker and PsR domain block the KaiC-CII binding site in the dimer. It should also be noted that without the PsR domain displacement, binding of the β-strand linker to KaiB would result in steric clashes of the PsR domain with neighboring KaiB subunits. It is currently not clear if the conformational change in KaiA is only required to sterically fit KaiA dimers to a KaiCB complex, or whether it indeed represents a functionally autoinhibited state that further prevents KaiA-stimulated autophosphorylation of KaiC beyond the sequestration mechanism.

Input and Output Protein Factors

We have discussed in detail how interactions between the Kai components produce circadian oscillations of KaiC autophosphorylation, but how does this signal translate to circadian rhythms of global gene expression? And how are environmental cues relayed to the circadian clock to ensure synchronicity between internal and external day-night cycles? The Kai system acts on global gene expression primarily as a suppressor of high amplitude promoters (Nakahira et al. 2004; Qin et al. 2010b). Kai is known to produce 24-h rhythms of chromosome compaction, which may act as a general mechanism to globally suppress transcription (Smith and Williams 2006; Woelfle et al. 2007). However, the output from the Kai system is also specifically targeted through signaling pathways that modulate transcription factors, which in turn regulate the multitude of sigma factors that are characteristic

of cyanobacteria. One of the foremost signaling pathways to mediate output from the Kai system consists of SasA, CikA, and RpaA (Iwasaki et al. 2000; Gutu and O'Shea 2013; Hertel et al. 2013; Kageyama et al. 2003; Markson et al. 2013; Nishiwaki et al. 2004; Takai et al. 2006). The circadian oscillation of Kai is relayed to the transcription factor RpaA through direct interactions of SasA and CikA with the Kai components. Following the circadian rhythms of KaiC autophosphorylation, these signaling components regulate global transcription in the cell, including feedback on the Kai components themselves (see Fig. 12.7a).

RpaA is a two-domain transcription factor, with a so-called response regulatory domain on the N-terminal half of the protein and an OmpR/PhoB-type DNA-binding domain on the C-terminal half. The response regulatory domain has a putative phosphorylation site at D53, which becomes phosphorylated by the histidine kinase SasA around subjective dusk (Gutu and O'Shea 2013; Hertel et al. 2013; Markson et al. 2013; Takai et al. 2006; Taniguchi et al. 2010). The SasA-RpaA interaction resembles that of the two-component signaling systems that are widespread in prokaryotes. Phosphorylation of RpaA follows the circadian rhythm of KaiC autophosphorylation with a -4-h phase shift. Phosphorylated RpaA binds directly to DNA approximately 20–50 base pairs upstream of the transcription

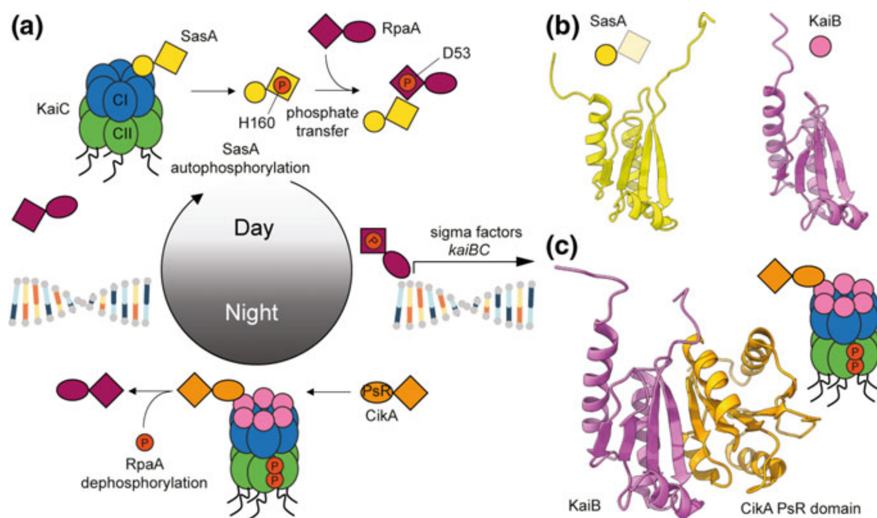

Fig. 12.7 The major output pathway of the Kai system. **a** Schematic of the SasA-RpaA-CikA system and interactions with Kai. During subjective day SasA binds to the same site as KaiB on KaiC-CI, resulting in autophosphorylation of SasA at H160. SasA autophosphorylation is relayed to transcription factor RpaA at D53. RpaA modulates transcription of many sigma factors and the *kaiBC* operon. During the subjective night, phosphorylated KaiCB complexes bind CikA resulting in dephosphorylation of RpaA, relieving its transcriptional regulation. **b** Ribbon diagrams of the thioredoxin-like folds of SasA (PDB ID 5JYU) and fold-switched KaiB (PDB ID 5JYT). Both domains bind to KaiC-CI, competing for the same binding site. **c** Ribbon diagram of KaiB in complex with the PsR domain of CikA (PDB ID 5JYV)

start sites, including the promoters of the *kaiBC* operon and the four sigma factor genes *rpoD2*, *rpoD5*, *rpoD6,* and *sigF2*. Some one hundred RpaA binding sites have currently been identified in the *Synechococcus elongatus* genome, at least half of which contain a characteristic A/T-rich motif. RpaA activates expression of a subset of its targets to peak at subjective dusk, whereas it *suppresses* activation of a different subset of targets to peak at subjective dawn. CikA gradually dephosphorylates RpaA during this phase of the circadian oscillator, thereby relieving RpaA's transcription activation by subjective dawn. RpaA further also regulates the transcription suppression activity of its paralogue RpaB (Hanaoka et al. 2012; Hertel et al. 2013). RpaB also binds to the promoters of the *kaiBC* operon and sigma factor genes *rpoD6* and *sigF2*, but at subjective dawn, suppressing their transcription. RpaB's displacement by RpaA further promotes expression of the circadian clock genes and downstream transcription regulators to produce circadian oscillations of global gene expression.

SasA and CikA relay the circadian oscillation of KaiC autophosphorylation to RpaA at subjective dawn and dusk, respectively. SasA is a two-domain histidine kinase that is responsible for phosphorylating RpaA at the putative D53 phosphorylation site (Markson et al. 2013; Takai et al. 2006). SasA has a thioredoxin-like domain on the N-terminal half of the protein and its kinase domain near the C-terminus (Vakonakis et al. 2004a). The thioredoxin-like domain is homologous to KaiB in the fold-switched, KaiC-CI bound state and it competes with KaiB for the same binding site (see Fig. 12.7b) (Murakami et al. 2012; Pattanayek et al. 2011; Tseng et al. 2014, 2017; Iida et al. 2015). SasA binds unphosphorylated KaiC at subjective dawn, resulting in autophosphorylation of SasA at H160 (Valencia et al. 2012). Autophosphorylated SasA dissociates from KaiC, oligomerizes, and subsequently relays its phosphate group to D53 of RpaA. As KaiC autophosphorylation levels rise and KaiCB complexes are formed, CikA binds to KaiCB complexes with its C-terminal Pseudo Receiver domain, interacting with the same β2 strand of KaiB that binds KaiA (see Fig. 12.7c) (Tseng et al. 2017). The RpaA-dephosphorylation activity of CikA is stimulated by the CikA interaction with KaiCB complexes (Gutu and O'Shea 2013).

Coupling of the Kai system with SasA-RpaA-CikA signaling modulates most physiological activity in cyanobacterial cells. Moreover, through SasA-RpaA-CikA signaling the Kai system also applies a gate on cell division (Dong et al. 2010; Yang et al. 2010). Whereas the inherent pace of the cell cycle is more or less independent from the circadian rhythm, the cell cycle does slow down during subjective night, when Kai suppresses cell division. Cyanobacteria can, therefore, divide multiple times per day, but only during a certain window set by the Kai system.

Feedback between the Kai oscillator and RpaA signaling regulates the circadian rhythm of the cell internally, but external signals also play a pivotal role in maintaining an adaptive biological clock. Both light and temperature are environmental cues that entrain the Kai system, which we will discuss in detail later. We would like to note here that even though light is such an important factor in cyanobacterial daily life, there is currently no known light-sensing protein that directly interacts with the

Kai oscillator. Rather, indirect effects from light via photosynthesis, such as the ATP/ADP ratio and redox state of the cell, are known to modulate the circadian rhythm of the Kai complex. The ATP/ADP ratio works directly on the KaiC ATPase and autophosphorylation activities, but input from the redox state of the cell requires at least two known sensing mechanisms. First, a redox-sensitive protein called LdpA interacts directly with KaiA and CikA (Ivleva et al. 2005). LdpA contains iron-sulfur clusters that are involved in sensing the redox state of the cell and the protein mediates depletion of KaiA and CikA under reducing conditions, effectively extending the period of the oscillation. In addition, KaiA and CikA are sensitive to the redox state of the cell through direct interactions of their PsR domains with oxidized plastoquinones that occur at the onset of darkness (Ivleva et al. 2006; Kim et al. 2012; Pattanayek et al. 2012; Wood et al. 2010). Quinone binding to KaiA or CikA results in aggregation and degradation of the proteins and causes a phase delay to the circadian rhythm of KaiC autophosphorylation.

Other known input and output pathways of the circadian clock operate mainly on the transcriptional level, with no known direct physical interactions with the Kai components. One of the earliest known examples of a transcriptional regulator of Kai is Pex (Arita et al. 2007; Kutsuna et al. 1998, 2007; Kurosawa et al. 2009). It forms a dimer with a winged-helix motif that recognizes a 25 base pair motif in the *kaiA* promoter region. Pex is expressed during prolonged dark periods and when it binds the *kaiA* promoter, it suppresses transcription. The net effect of Pex is to extend the period of the circadian oscillator. Other genes that are known to be involved with transcriptional regulation of the circadian clock include *labA*, *lalA*, *cpmA,* and *crm*, which act on either *kaiA* or *kaiBC* promoters. However, molecular details of these interactions still remain elusive (Boyd et al. 2013; Katayama et al. 1999; Taniguchi et al. 2001, 2007, 2010).

Feedback Mechanisms and Entrainment

The negative feedback loop arising from the self-repression of a gene by its gene product is a common motif found in prokaryotic as well as eukaryotic oscillators. Powerful oscillations arise as soon as the feedback loop contains a certain delay. Negative feedback loops lie at the core of many rhythmic processes within cells (DeWoskin et al. 2014), including those involved in circadian timekeeping, embryogenesis, cell cycle, and DNA damage repair providing several major advantages: temporal organization, spatial organization, prediction of repetitive events, efficiency, and precision of control (Rapp 1987).

Circadian clocks responsible for endogenous 24-h oscillations are divided into two groups in regard to their functionality, either based on a transcription-translation feedback loop (TTFL) or a post-translational oscillator (PTO). Eukaryotic circadian clocks are mainly based on TTFL, whereas cyanobacterial circadian clocks, as the only known example for prokaryotic circadian clocks, comprise a PTO (Johnson et al. 2011). Protein sequence analyses of the clock components

suggest a convergent evolution with multiple origins of circadian clocks, which is supported by the different functionality of known circadian clocks (Johnson et al. 2011; Rosbash 2009).

A decade of intensive research on the mechanisms and functionality of the KaiABC system—the cyanobacterial PTO-based clock—using computational and mathematical approaches in addition to the detailed biochemical and biophysical understanding make this the best understood circadian clock. Various parts of the KaiABC system were identified and described in detail through computational modeling: The ordered phosphorylation of KaiC and temperature compensation of the clock (Brettschneider et al. 2010; Rust et al. 2007), the stimulating interaction with the other core factors and effects on gene expression (Clodong et al. 2007; Kurosawa et al. 2006; van Zon et al. 2007), as well as the influence of varying ATP/ADP ratios (Rust et al. 2011). Further, mathematical models have identified different strategies for period robustness against internal and external noise, reviewed in Schmelling and Axmann (2018).

The remarkably robust high-amplitude phosphorylation cycles are stabilized by an intracellular synchronization of different KaiC hexamers: This is on the one hand achieved by an exchange of KaiC monomers (Emberly and Wingreen 2006; Mori et al. 2007; Yoda et al. 2007). On the other hand, mathematical modeling demonstrated that sequestration of free KaiA mediates synchronization (Brettschneider et al. 2010; Clodong et al. 2007; Qin et al. 2010a; van Zon et al. 2007). Together, synchronization by KaiA-sequestration and KaiC-monomer exchange leads to robust intracellular oscillations. In addition, the three-protein clock is embedded in a TTFL, similar to eukaryotic clock systems (Hertel et al. 2013; Zwicker et al. 2010). A two-loop transcriptional feedback mechanism could be identified in which only one phosphorylation form of KaiC suppresses *kaiBC* expression while two other forms activate its own expression. In particular: KaiC-S/pT and KaiC-pS/pT activate and KaiC-S/T suppress *kaiBC* transcription (Hertel et al. 2013). Due to this further layer of control, the PTO becomes insensitive to the manifold perturbations in living cells; reviewed in Johnson and Egli (2014).

Besides the circadian clock as seen in *Synechococcus elongatus*, which functions similar to a limit cycle oscillator (Gan and O'Shea 2017; Pittayakanchit et al. 2018), there exists a reduced form of timing mechanism in cyanobacteria, as described before: An hourglass timing system as seen in *Prochlorococcus*, which works more like a point attractor that stops oscillating and relaxes to a stable fixed point in the absence of an exogenous cycle (Pittayakanchit et al. 2018). Fluctuations of external cues have different effects on the circadian clock systems in cyanobacteria. Whereas, the limit cycle oscillator (*Synechococcus*) is almost unaffected (Paijmans et al. 2017; Pittayakanchit et al. 2018), the point attractor (*Prochlorococcus*) is set in free fall towards the night state (Pittayakanchit et al. 2018). On the other side, when considering only internal noise due to finite numbers of proteins the point attractor outperforms the limit cycle in regard to precision due to its ability to change faster between both states (Pittayakanchit et al. 2018).

One defining characteristic of a circadian clock is the ability to be entrained to an exogenous cycle. Thus, a circadian system can be synchronized to an exogenous

stimulus in order to be most useful for the organism in anticipating recurring patterns. However, the clock also has to be robust against naturally occurring fluctuation of the input signal. There are two strategies for entraining the clock to an exogenous stimulus: (i) direct sensing of light, a strategy that is normally used by eukaryotic circadian clock systems (Rosbash 2009) and (ii) indirect sensing of light through changes in the metabolic state of the cell, i.e. redox state or ATP/ADP ratio, which is used by cyanobacteria (Rust et al. 2011; Shultzaberger et al. 2015). It has experimentally been shown that the phase of the circadian clock is affected by the ATP/ADP ratio, which is a result of the cellular catabolic metabolism and the photosynthetic apparatus (Shultzaberger et al. 2015). Dark phases cause a drop in the ATP/ADP ratio, which shifts the clock into the dephosphorylation phase. Even the in vitro clock reacts differently to the ATP/ADP ratio (Rust et al. 2011). During the phosphorylation phase (subjective day), the oscillator is most susceptible to changes in the ATP/ADP ratio, whereas in the dephosphorylation phase (subjective night) the oscillator is almost insensitive. The underlying mechanism for this phase shift might be explained by the ADP/ATP exchange in the CII domains that changes during the cycle (Nishiwaki-Ohkawa et al. 2014). Thus, the entrainment mechanism for the KaiABC clock works in vitro and does not rely on an additional signaling pathway, resembling the direct effect of the metabolic state of the cell on the phase of the circadian clock (Rust et al. 2011).

Diversity and Evolution of Kai-Protein System

The unique three-protein oscillator is well documented for the model cyanobacterium *Synechococcus elongatus* PCC 7942. The complex formation between the Kai proteins has been discovered in an exquisitely high resolution emphasizing the importance of the stoichiometry between the three Kai proteins in maintaining robust circadian oscillations. Thus, it is puzzling that several cyanobacteria, e.g. *Synechocystis* sp. PCC 6803, a model organism for photosynthesis and industrial applications, contain multiple *kai*-gene copies in the genome (Schmelling et al. 2017; Wiegard et al. 2013). Other cyanobacteria like marine *Prochlorococcus* strains harbor only a reduced *kaiBC* system, thus, lacking the *kaiA* gene.

Cyanobacteria represent one of the most diverse bacterial phyla regarding their genomes, morphology, and physiology, which is reflected by the diversity of their timing systems. The enormous amount of available genomic data allows a comprehensive overview of the distribution and conservation of the clock factors. Surprisingly, orthologs for multiple clock components, including the core factors KaiB and KaiC, are present in a large number of bacterial and archaeal genera (Schmelling et al. 2017; Dvornyk et al. 2003, 2004). However, the presence of *kaiA* in the genome is absolutely restricted to Cyanobacteria. The high conservation of motifs for the interaction of KaiA and KaiB in Cyanobacteria, where the β2 sheet represents the most conserved part of the KaiB protein (Snijder et al. 2017), underlines the special role and evolution of KaiA being the key component for a

true circadian clock. Even in light of the huge diversity of timing systems among Cyanobacteria, two protein sets can be suggested: (i) factors that are found in almost all cyanobacteria, especially KaiB, KaiC, LdpA, SasA, RpaA, and RpaB, indicating that those factors comprise the core set needed for timing; (ii) factors only found in a set of cyanobacteria like KaiA, CikA, and LabA which are important for circadian regulation in *Synechococcus elongatus* PCC 7942 (Schmelling et al. 2017). *Prochlorococcus* strains are lacking genes for KaiA, CikA, and LabA (Axmann et al. 2009). They show stable oscillations of gene expression under light-dark cycles, however, these oscillations disappear under continuous conditions (Holtzendorff et al. 2008). Thus, the reduced KaiBC-timing system in *Prochlorococcus* is not considered as a circadian clock, since oscillations cease without an exogenous stimulus. Instead, it is called an hourglass-like clock (Axmann et al. 2009).

Kai proteins as well as in- and output factors of the clock are well conserved among Cyanobacteria, however, they are also present in other bacteria and Archaea (Schmelling et al. 2017). Interestingly, they share no sequence similarity to any known eukaryotic clock component. In general, the presence of homologous genes decreases from Cyanobacteria to Proteobacteria to Archaea. However, the key component KaiA, as well as the input factors Pex, LdpA, and CdpA, are absent outside the cyanobacterial phylum (Schmelling et al. 2017). Although known input factors are absent in non-cyanobacteria and Archaea, there might exist input pathways enabling entrainment of the endogenous timing system with the environment. A direct input by the ATP/ADP ratio might be a primary mechanism to transmit changes and synchronize the inner timing system with the internal metabolism and the external environment (Schmelling et al. 2017). Further different but species-specific input factors might exist. In the photoheterotrophic proteobacterium *Rhodobacter sphaeroides* for example, which displays 24-h gene expression rhythms, a histidine kinase is encoded in an operon with *kaiBC* and was suggested as a candidate for transducing the redox signal to KaiBC (Min et al. 2005).

Reduced KaiBC-based or even simpler, solely KaiC-based timing systems might exist in other bacteria and Archaea. For example, in the halophilic archaeon *Haloferax volcanii* the transcripts of four *kaiC* homologs display diurnal accumulation profiles, and those profiles are abolished by deletion of one of the *kaiC*-like genes (Maniscalco et al. 2014). Whether the encoded KaiC proteins in Bacteria and Archaea are able to measure time via phosphate uptake can be elucidated by biochemical approaches. Analyzing the kinase activity of KaiC using purified proteins from two hyperthermophilic Archaea, *Thermococcus litoralis* and *Pyrococcus horikoshii*, and several cyanobacterial species demonstrated that kinase activity of distantly related KaiC proteins is well conserved (Schmelling et al. 2017).

Regarding the evolution of KaiC, two valid hypotheses exist, both of which state that KaiC arose from a shorter ancestral *recA* gene followed by gene duplication and fusion (Dvornyk et al. 2003; Leipe et al. 2000). However, on the one hand, it is hypothesized that an ancestral single-domain KaiC originated in Bacteria, was transferred into Archaea, where its two-domain version originated, and a second lateral transfer event introduced the double domain KaiC into cyanobacteria

(Leipe et al. 2000). On the other hand, it is argued that KaiC has to be of cyanobacterial origin (Dvornyk et al. 2003). Given the amount of new genomic data, further studies are promising to unravel the evolutionary history of KaiC in the near future.

Among the three *kai* genes, *kaiC* is evolutionarily the oldest, and *kaiA* is the youngest. A major event was the origin of the *kaiB* gene and the formation of the *kaiBC* cluster between 3,500 and 2,320 million years ago (Dvornyk et al. 2003). This time corresponds to the period when a reducing geochemical environment, which had existed from the beginning on, was replaced by an oxidizing environment produced by cyanobacteria themselves. Given that *kaiA* can be found today in cyanobacteria only, it is very likely that it evolved there as well. Marine *Prochlorococcus* species lost *kaiA* during evolution. The three-gene *kaiABC* cluster itself evolved around 1,000 million years ago—a key event in the evolution of cyanobacteria, which ensured their domination in Earth's ecosystems (Dvornyk et al. 2003).

Circadian clocks appear to be a conserved trait in evolution providing a fitness advantage by two directions: by synchronizing processes to external, environmental factors, and by coordinating internal processes optimally. Predicting repetitive events provides a major advantage for organisms enabling higher efficiency and precision of control. Summarizing our knowledge on circadian clocks suggests a convergent evolution. The invention of time-keeping mechanisms occurred several times independently in evolution and gave rise to diverse clockworks and timers. However, reports on ubiquitous metabolic cycles accumulated in the last years constituting a universal marker for circadian rhythms in all domains of life, Bacteria, Archaea and Eukaryota. An intimate co-evolution of cellular timekeeping has been suggested with redox homeostatic mechanisms after the Great Oxidation Event about 2.5 billion years ago (Edgar et al. 2012), when photosynthetic cyanobacteria started to evolve oxygen from water increasing atmospheric oxygen dramatically. Rhythms of photosynthetically produced oxygen and corresponding reactive oxygen species (ROS) made by sunlight might be a key driving force in the coevolution of the clock and ROS removal systems that would enable anticipation and resonation with externally driven redox cycles (Reddy and Rey 2014). However, many questions remain on how evolution invented time-measuring systems, and what selective pressures were needed to create a clock.

Outlook

The physiological diversity and genetic accessibility of phototrophic cyanobacteria has recently attracted growing attention to using them as promising biological chassis for the synthesis of a variety of natural products. This includes bioactive metabolites like cytotoxins and potential pharmaceutical lead compounds, food supplement, animal feed, pigments, as well as biofuels (Chlipala et al. 2011; Knoop and Steuer 2015; Loeschcke et al. 2017). Due to the ability of cyanobacteria to convert sunlight and atmospheric CO_2 directly into valuable organic compounds

like sugars, they could provide sustainable alternatives to fuel biotech processes (Ducat et al. 2011; Hays et al. 2017; Weiss et al. 2017). Moreover, cyanobacteria do not compete for arable land or drinking water—they can be cultivated in open ponds and space-saving bioreactors. This could be of global importance and solve one of the biggest problems humanity is facing in the 21st century—worldwide CO_2 emissions are increasing while natural carbon and drinking water resources are being exhausted.

Understanding how we could control and optimize metabolic processes in light-dark cycles will be important for further establishing phototrophic organisms like cyanobacteria and microalgae in biotechnological applications (Welkie et al. 2019). Indeed, the circadian clock influences metabolism drastically. In particular, RpaA seems to critically influence growth rates by 60% (Ungerer et al. 2018). Thus, manipulating components of the circadian clock leads to higher productivity regarding biotechnologically relevant products (Osanai et al. 2015).

Further, the cyanobacterial KaiABC clock is transferrable to a heterologous organism, which could be demonstrated for *E. coli* (Chen et al. 2015), opening synthetic circadian design in a broad range of organisms. Overall the insights gained by studying the KaiABC clock of cyanobacteria have substantial impact on the field of circadian research, which can be used for the design of synthetic switches, oscillators, and clocks in the future.

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