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Principles of rhythmicity emerging from cyanobacteria

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Over the past 25 years the cyanobacterium *Synechococcus elongatus* has dazzled the circadian rhythms community by revealing in exquisite detail the mechanism of its prokaryotic circadian clock. So many aspects of the timing machinery are surprising that attention has largely centered on the ways in which the cyanobacterial clock is different from the circadian clocks of eukaryotic organisms. Perhaps just as remarkable is the similarity of circadian properties between eukaryotic and prokaryotic species – a testament to the universality of unfaltering daily cues in the environment and shared metabolic needs of biological systems as selective agents over evolutionary time. Like the clocks of animals and plants, the *S. elongatus* clock supports near-24-h rhythms that persist in constant conditions, entrains to daily cycles of light or temperature, is differentially sensitive to cues at different points in the cycle, and tunes its daily period depending on the intensity of the ambient light environment. Remarkably, it supports these properties with a nanomachine mechanism that is discrete and can be reassembled to function outside of the cell. The lessons learned from the *S. elongatus* clock underscore the power of genetics to reveal mechanisms whose natures are not known *a priori*, and speak to the value of collaboration to apply diverse skillsets to solve difficult biological problems.

Emergence of a cyanobacterial circadian model In the early 1990s few gave much thought to bacteria as circadian model systems. Their typical unicellular and fast-growing lifestyles, and simple cellular structure, led most to assume that a circadian clock would not be of evolutionary value, or even supportable, in bacterial cells. Evidence scattered throughout the literature had hinted at an endogenous clock in various genera of cyanobacteria (Golden *et al.*, 1997). Finally, the group of T-C Huang (Academia Sinica, Taiwan) produced convincing reports that some physiological processes in a cyanobacterium fit the criteria accepted in eukaryotes as evidence of circadian control (Huang *et al.*, 1990; Chen *et al.*, 1991). However, the reported observations of rhythmic photosynthetic activity, nitrogen fixation, and amino acid uptake were not amenable to mechanistic analysis. Elevating the observations from phenomenology to a biological system worthy of study would require the ability to uncover components of the timing mechanism. The few eukaryotic clock genes known at that time did not yield hybridization signals with cyanobacterial genomic DNA, leaving the cyanobacterial clock as a black box that would require a forward genetic screen to open. Enabling this approach required: the slight leap of faith that another cyanobacterial species, more easily manipulated than those with published circadian phenotypes, would also have a clock; the genetic know-how of working with a cyanobacterial model system; and, the ability to design a bespoke monitoring device. By 1993 a screenable phenotype was demonstrated: a *bona fide* circadian rhythm of luciferase reporter gene expression in *Synechococcus elongatus* (Kondo *et al.*, 1993).

Luciferase-based bioluminescence rhythms revealed that the circadian properties of the *S. elongatus* clock are essentially indistinguishable from those of eukaryotic systems. Circadian rhythms persist with a period of approximately 24 h when cells are kept in continuous light and temperature, and the relative phase of the rhythm can be reset by a pulse of darkness (Kondo *et al.*, 1993), with the magnitude of the resulting phase shift dependent on the point in the cycle at which it is applied (Schmitz *et al.*, 2000). The period is temperature compensated, and cycles of a few degrees of change in temperature, applied as artificial dawn and dusk, can entrain the clock (Yoshida *et al.*, 2009). Moreover, the cyanobacterial clock adheres to “Aschoff’s Rule,” such that the free-running period shows a dependence on light intensity, trending shorter with higher light as is true for diurnal eukaryotes (Katayama *et al.*, 2003). Unlike neurons of the suprachiasmatic nucleus, there is no evidence of coupling among cells in a population (Mihalcescu *et al.*, 2004). The robust, coherent rhythms exhibited by samples of millions to billions of cells rely on cell-autonomous “atomic clocks” that, once set, are inherited with fidelity at each cell division.

The Kai-complex nanomachine Fast-forward 25 years to the present, and appreciate the remarkable progress that has been made since a screen for mutants defective in bioluminescence from luciferase reporters (Kondo *et al.*, 1994) led to the discovery of the genes *kaiA*, *kaiB*, and *kaiC*, which underpin the circadian mechanism in *S. elongatus* (Ishiura *et al.*, 1998). The proteins that these genes encode had not been described previously and held few clues as to how they contribute to a circadian clock. The combined efforts of geneticists, biochemists, and structural biologists over a decade led to a clear picture of an elegant nanomachine whose basic timing loop can be reconstituted *in vitro* (Nakajima *et al.*, 2005; Swan *et al.*, 2018). The *in vitro* oscillator stands out as a fascinating achievement in biochemistry, with implications well beyond the realm of circadian

biology. In a simple mixture that contains the KaiA, KaiB, and KaiC proteins, a 24-h oscillation of protein states is established that can be monitored by the rhythmic autophosphorylation and dephosphorylation of KaiC (Nakajima *et al.*, 2005) or the change in fluorescence polarization of a tagged component as it associates and disassociates from the larger complex (Leypunskiy *et al.*, 2017).

Many unforeseen steps contribute to the timekeeping mechanism, in which KaiC plays a central role as the foundation structure on which other components dock. The simplest description of the oscillator is that KaiC autophosphorylates when stimulated to do so by the binding of KaiA, and autodephosphorylates when KaiB associates and, thereby, blocks the stimulatory action of KaiA (Swan *et al.*, 2018). More detailed structural investigation has revealed intra- and inter-protein interactions that contribute to timing, as each interaction makes the forward tick of the clock hand more favorable and a backwards tick less so. An animation that accurately describes key structural steps in the cycle is available online through the BioClock Studio (Nguyen *et al.*, 2016a).The hexameric repeat-domain structure of KaiC results in the creation of two rings, one stacked on the other, that take turns being more loosely or tightly hexamerized, depending on the phosphorylation state of two residues in the C-terminal domains (CII ring), and whether ATP has been hydrolyzed by the N-terminal CI domains (Chang *et al.*, 2011, 2012; Tseng *et al.*, 2017). These changes affect how tightly the rings stack upon one another, and determine the accessibility of binding sites for KaiA and KaiB. KaiA has been known for 15 years to bind to the C-terminal tails that dangle from CII, stimulating KaiC to autophosphorylate (Kim *et al.*, 2008). More recent work showed that KaiA itself oscillates in structure between an active form that carries out the stimulation of KaiC phosphorylation, and an inactive form that is trapped by KaiB (Tseng *et al.*, 2017). KaiB was recently discovered to spend most of its time in an inactive tetrameric state that does not interact with the other Kai proteins. Rarely, the tetramer dissociates and the polypeptides unravel, refolding in an entirely different tertiary structure (Chang *et al.*, 2015). This “fold-switched” KaiB is the active form; it is unstable, and quickly reverts to the inactive ground state unless it finds its binding site on KaiC. Once bound to KaiC, it forms a third ring on the complex, which serves as a hub for a “night-time complex” (Tseng *et al.*, 2017). The KaiB ring captures and sequesters the inactive state of KaiA, initiating the dephosphorylation phase of KaiC and preparation for the dawn phase of the cycle. Although this three-some of Kai proteins is sufficient to establish a robust circadian rhythm, at least two other components physically interact with the Kai complex and participate in the regulation of circadian period: the histidine protein kinases SasA (Iwasaki *et al.*, 2000) and CikA (Schmitz *et al.*, 2000).

Input, oscillator, and output Another feature of elegance in the cyanobacterial clock is the integration of mechanisms that mediate entraining input and temporal output into the oscillator complex itself, as is true to various extents in eukaryotic clocks as well. One variation is that the cyanobacterial clock does not seem to employ a sensory photoreceptor-based signal transduction pathway to entrain its mechanism, but rather uses the metabolic products of the cell’s photosynthetic machinery to determine when the lights are on (Rust *et al.*, 2011; Kim *et al.*, 2012). Changes in the ratio of ATP to ADP that occur upon lights on and off affect the binding of these nucleotide species by KaiC, influencing those hexamer-tightness and ring-ring interactions of KaiC that determine interactions with other clock proteins. In addition, both KaiA and CikA have domains that bind specifically the

oxidized form of quinones (Ivleva *et al.*, 2006; Wood *et al.*, 2010), which are key players in photosynthetic electron transport and are sensitive to redox changes that accompany light-dependent dynamics. Quinone binding affects the association of each of these proteins with the Kai complex, and hence changing the chemical time stamp for phase in the cycle. Indeed, manipulation of the ATP/ADP ratio or the addition of oxidized quinones to the *in vitro* oscillator can reset the phase of the KaiC phosphorylation rhythm with the same properties observed for dark-pulse resetting of gene expression rhythms *in vivo* (Rust *et al.*, 2011; Kim *et al.*, 2012).

The components that relay temporal information of the oscillator status to downstream gene expression are also fundamentally part of the Kai complex (Tseng *et al.*, 2014, 2017). Although CikA's role in the clock was discovered through its necessity for phase resetting (Schmitz *et al.*, 2000), later work showed that it plays a critical role in circadian output as well (Gutu & O'Shea, 2013). Moreover, CikA competes with KaiA for binding to the KaiB ring in the night-time complex (Tseng *et al.*, 2017; Welkie *et al.*, 2018). Hence, CikA can be considered part of the oscillator itself, affecting circadian period both *in vivo* and *in vitro*. The circadian output role of CikA depends on a phosphatase activity that is triggered by engagement of CikA with the Kai complex at night (Gutu & O'Shea, 2013; Welkie *et al.*, 2018). This activity dephosphorylates a master transcription factor, RpaA, whose rhythmic phosphorylation sets up the oscillation of gene expression that is observable through the entire genome to various extents. The phosphorylation of RpaA is also clock-controlled, being stimulated by another kinase called SasA, which binds directly to KaiC on a site that overlaps with the KaiB-binding site, and is likely displaced by KaiB when the latter forms its ring after dusk (Iwasaki *et al.*, 2000; Takai *et al.*, 2006; Gutu & O'Shea, 2013; Tseng *et al.*, 2014, 2017). SasA kinase activity towards RpaA is stimulated only when SasA is engaged with the Kai complex. Hence, rhythmic waves of RpaA phosphorylation depend on the temporal separation of SasA kinase and CikA phosphatase activities. Like CikA, SasA can be considered an integral part of the oscillator *in vivo*, and its presence affects circadian period (Iwasaki *et al.*, 2000). An animation that explains how interactions of CikA and SasA with the Kai complex result in rhythmic transcription of the genome is available online through the BioClock Studio (Nguyen *et al.*, 2016b).

Different ways to build a clock The basic timing system of the *S. elongatus* clock does not depend on a transcription-translation feedback loop, the fundamental model for eukaryotic clocks (Bell-Pedersen *et al.*, 2005). The persistent, temperature-compensated, resettable properties of the *in vitro* Kai oscillator make this statement irrefutable (Nakajima *et al.*, 2005). Some studies have shown that the regulation of the *kaiBC* operon by RpaA, and hence by clock output, is an important reinforcing loop that stabilizes the rhythm (Qin *et al.*, 2010; Teng *et al.*, 2013). However, even *in vivo*, the Kai oscillator can run a very respectable circadian gene expression program when the *kai* genes are expressed from a heterologous RpaA-independent promoter or not transcribed at all (Tomita *et al.*, 2005; Markson *et al.*, 2013). Hence, the blueprints for the cyanobacterial and known eukaryotic clocks are simply different. The complete alignment of circadian properties, despite a fundamental difference in mechanism, speaks to the universal importance of these clock properties for environmental fitness, and the nature of evolution as, quite literally, a Blind Watchmaker (Dawkins, 1986).

The prokaryotic and eukaryotic clock blueprints are not without similarities. Phosphorylation plays a key role in determining the temporal status of progression through the circadian cycle in all known systems, although the consequence of the conformational change it imparts varies from component to component (Bell-Pedersen *et al.*, 2005; Hardin & Panda, 2013; Hurley *et al.*, 2016). In diverse systems the molecular circuits that comprise oscillators are sensitive to the metabolic status of the cells that support them, such that some metabolites serve as time cues that are as powerful as light in some cell types (Hatori & Panda, 2015; Frank *et al.*, 2018). Because the cyanobacterial oscillator runs so well in *in vitro*, and in the absence of transcription or translation *in vivo*, the potential role of proteolysis, known to be important in eukaryotic clocks (Hurley *et al.*, 2016), has been largely ignored in the bacterial system. However, it turns out that the major protease in *S. elongatus*, ClpXP, affects circadian period and limits the range of phase change that can occur in a single cycle (Cohen *et al.*, 2018). The specific targets that are regulated by proteolysis are not yet defined, but some evidence points to KaiC itself as a ClpXP substrate. The robust *in vitro* oscillator also makes it tempting to envision *S. elongatus* cells as green test tubes, in which proteins diffuse randomly. In reality, prokaryotic cells have extensive ultrastructural compartmentalization, albeit more difficult to visualize and less investigated than the membrane-bound organelles and cytoskeleton of eukaryotic cells (Surovtsev & Jacobs-Wagner, 2018). The clock components in *S. elongatus* localize near a pole of the cell rhythmically, with maximal localization at night, in a clock-dependent manner (Cohen *et al.*, 2014). This subcellular localization is reminiscent of the cytoplasmic-to-nuclear changes of eukaryotic oscillator components, such as Period and its binding partners (Saez *et al.*, 2007). Whereas the regulation by subcellular localization of eukaryotic clock components is known and has been extensively studied, neither the structural determinants of localization, nor the role of localization in circadian function, is understood in the cyanobacterium at this time.

What have we learned? Given the basic difference in timekeeping machinery between cyanobacteria and neurons, one might ask what instructive lessons for human circadian biology follow from investigation of the Kai clock. One major inroad was the clear demonstration in the cyanobacterial system that our notion of circadian clocks as fitness engines is indeed correct. The ability to grow cyanobacterial strains whose intrinsic periods differ showed conclusively that a resonance between the internal clock and an external day-night cycle confers a fitness advantage (Ouyang *et al.*, 1998). The most important insight from the cyanobacterial system may be the great expansion of the range of possibilities of how a clock might be built, to encourage researchers to reach beyond expectations that are based on prior knowledge. The biggest advances in the cyanobacterial circadian system were realized when the investigators accepted that preconceived notions were incorrect, shed hypotheses, and turned to unconventional approaches. Notable examples include the audacious undertaking by the T. Kondo laboratory to attempt *in vitro* reconstitution of a circadian oscillator (Nakajima *et al.*, 2005), and the crucial participation of structural biologists to apply a variety of methods to gain insights into the functions of proteins that lacked bioinformatic signposts (Williams *et al.*, 2002; Garces *et al.*, 2004; Iwase *et al.*, 2004; Pattanayek *et al.*, 2004; Ye *et al.*, 2004; Hitomi *et al.*, 2005; Chang *et al.*, 2012). The rare fold switch of KaiB was as unexpected and as mysterious a step in the timing loop as one could imagine, and it was discovered only through a combination of biophysical measurements –

nuclear magnetic resonance and electron paramagnetic resonance – that are entirely arcane to most circadian biologists (Chang *et al.*, 2015). Once discovered and stabilized, fold-switched KaiB rendered Kai complexes amenable to X-ray crystallography (Tseng *et al.*, 2017).

The real extent of diversity of clock mechanisms in nature is unknown, because so few organisms, and in such narrow phylogenetic clades, have been investigated. To discover the true universe of circadian mechanisms, it may be necessary to abandon the security of investigating only systems in which rhythms persist in constant conditions. Such a criterion is unlikely to be important for fitness outside of the lab, or to the Blind Watchmaker of evolution. Even among prokaryotes, there is evidence for other clocks (Edgar *et al.*, 2012; Ma *et al.*, 2016; Paulose *et al.*, 2016), but so far no other system is as robust and approachable for genetic analysis as *S. elongatus*, rendering all much more challenging to understand.

Can we reject the notion that a nanomachine clock may be fundamental to circadian rhythms in the well-studied eukaryotic systems? The known components of transcription-translation feedback loops are necessary for self-sustained rhythms, but it is difficult to exclude an unforeseen oscillator that works on a different blueprint and which could be as central to the ultimate timing loop as the parts we know of. If such exists, it likely comprises elements that are essential for viability, or else would have emerged through arrhythmic mutants in exhaustive genetic screens. Moreover, a mechanism could be necessary, yet must be insufficient, such that the rhythms are lost without the known loops. The rapid advance of technologies that enable systems-level analysis, high throughput, and computational deconvolution of data may lead to the discovery of even more ways that nature has built a clock.

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