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A circadian clock nanomachine that runs without transcription or translation

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The biochemical basis of circadian timekeeping is best characterized in cyanobacteria. The structures of its key molecular players, KaiA, KaiB, and KaiC are known and these proteins can reconstitute a remarkable circadian oscillation in a test tube. KaiC is rhythmically phosphorylated and its phospho-status is a marker of circadian phase that regulates ATPase activity and the oscillating assembly of a nanomachine. Analyses of the nanomachines have revealed how their timing circuit is ratcheted to be unidirectional and how they stay in synch to ensure a robust oscillator. These insights are likely to elucidate circadian timekeeping in higher organisms, including how transcription and translation could appear to be a core circadian timer when the true pacemaker is an embedded biochemical oscillator.

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Introduction

The core mechanism of the circadian clock in eukaryotic cells is widely held to be based on a Transcription/Translation Feedback Loop (TTFL) [1,2], although there is recent evidence that this model may be incomplete or inaccurate [3,4]. In cyanobacteria, the initial evidence also supported a TTFL model [5]. However, our current understanding of the clock system in cyanobacteria is that a biochemical oscillator operates as the central 'quartz crystal' of the clockwork [6••], but this core pacemaker operates within (and regulates) a larger TTFL that controls outputs and replenishes the oscillator's essential proteins [7,8••]. The remarkable discovery that this core oscillator could be transplanted as a three-protein system to oscillate *in vitro* [6••] has led some researchers to revisit the question of non-TTFL circadian clocks in eukaryotes — a search that has recently culminated in the discovery of circadian metabolic/redox oscillations that can operate in eukaryotes in the absence of transcription [9], and which resurrects an old literature on circadian clocks in enucleated algal cells [4]. Because we know the 3D-structures of the major protein players and the oscillator can be reconstituted *in vitro*, the cyanobacterial system constitutes a unique preparation to study the biochemistry, biophysics, and structural biology of post-translational circadian timekeeping.

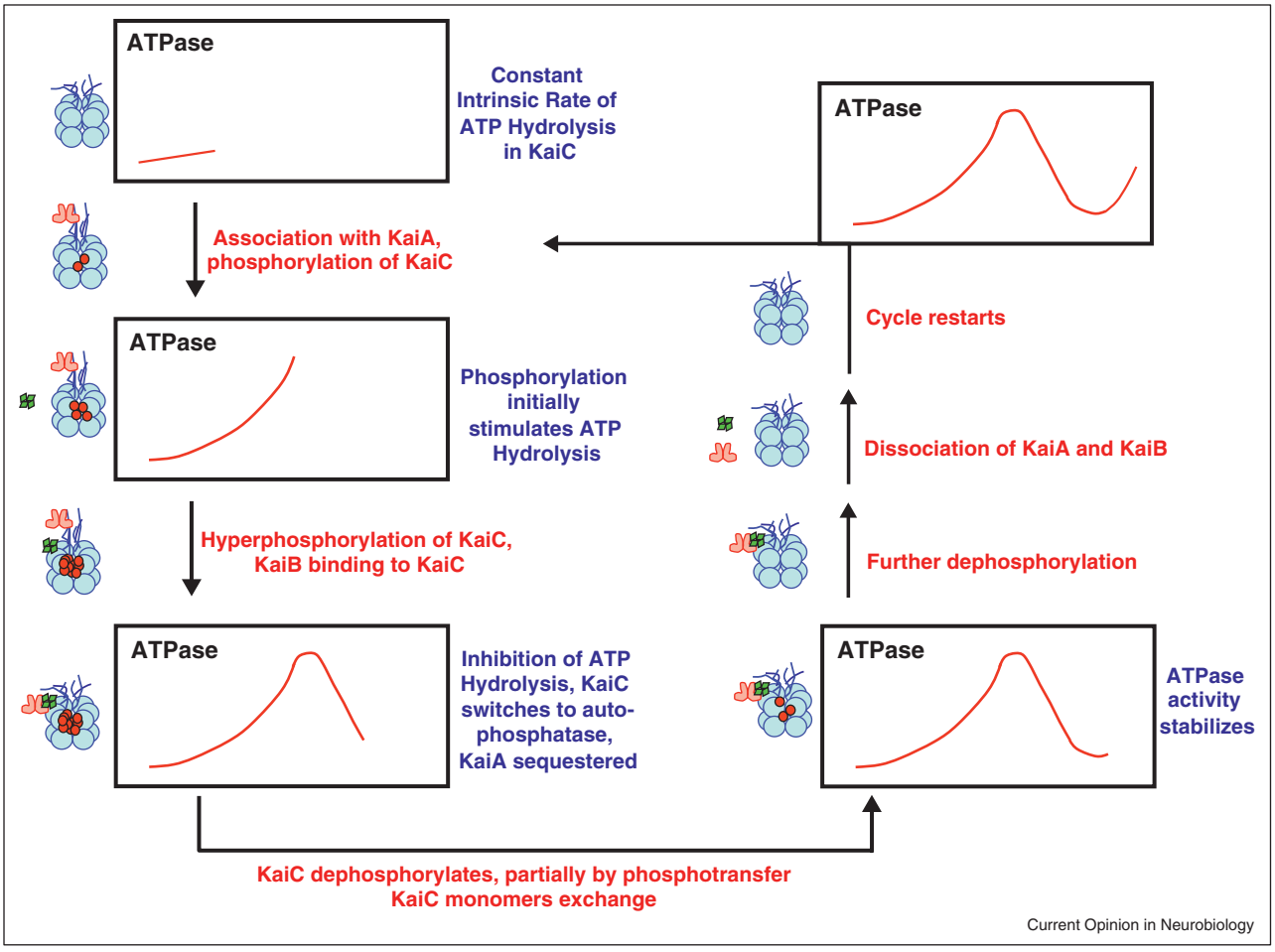
To set the stage for the information that we summarize below, KaiC hydrolyzes ATP and this reaction may be an intrinsically constant-rate timer [10] that drives KaiC phosphorylation. The status of KaiC phosphorylation feeds back to regulate first, the ATP hydrolytic rate (so that its intrinsic constant rate becomes rhythmic) and second, switching of the KaiABC nanocomplex between autokinase and autophosphatase states (Figure 1). An overly simplified analogy would be to consider KaiC ATPase activity as an 'hourglass timer' which is restarted each cycle (the 'hourglass is turned over') by KaiC's phosphorylation status, thereby converting the hourglass timer into an oscillation. Therefore, the extent of KaiC phosphorylation is both a marker of circadian phase [11,12] and a regulator of KaiC's myriad activities (ATPase, autokinase, autophosphatase, phosphotransferase, and nanocomplex formation).

Architecture and function of KaiC, the central player

The N-terminal and C-terminal domains (termed CI and CII, respectively) of KaiC exhibit sequence similarity with ATPases, such as the DnaB helicase and RecA recombinase. The crystal structure of *Synechococcus elongatus* KaiC resembles a double doughnut with CI and CII rings of similar height and width and a constricted waist region that is spanned by 15-amino acid linkers (Figure 2a) [13]. The conformation of the C-terminal region in CII differs considerably from the one in CI that constitutes the linker and winds along the outer surface of the CI/CII interface. Thus, CII C-terminal peptides form a crown-like arrangement of S-shaped loops ('tentacles'), comprised of residues E487 to I497, that rim the central KaiC channel and then protrude from the outer dome-shaped surface of CII.

ATP molecules are wedged between subunits in the CI and CII rings and bound to P-loop motifs (Figure 2) [13]. The two rings possess different functions; the CI ring

Figure 1



The circadian *in vitro* oscillator that acts as a post-translational oscillator (PTO) *in vivo*. The reaction is composed of ATP hydrolytic activity and phosphorylation within KaiC, and rhythmic associations of KaiC with KaiA and KaiB to form a nanocomplex that regulates ATPase and phosphorylation activities. Cartoons depict associations among the KaiC hexamer (light blue), the KaiA dimer (pink), the KaiB tetramer (green) and phosphorylation on KaiC subunits (red dots). Rectangles show ATPase activity over time. Starting from the upper left corner, KaiA associates with unphosphorylated KaiC and stimulates autophosphorylation, which accelerates the rate of ATP hydrolysis. The stages of the reaction proceed as described in the text [41•] in a counter-clockwise direction.

harbors ATPase activity [10] and the CII ring catalyzes rhythmic phosphorylation and dephosphorylation reactions. The kinase activity [14,15] results in phosphorylation of Thr432 and Ser431 across CII subunit interfaces [13,16]. A third residue, T426, influences phosphorylation and dephosphorylation of T432/S431, possibly by acting as a labile phosphorylation site whereby a phosphate is shuttled between T426 and S431 [16,17]. The T432 residue is phosphorylated first in the cycle, followed by phosphorylation at S431 [11,12], and this strict order of phosphorylation can be explained with a kinetically controlled kinase activity, where the ATP γ-phosphate is closest to the hydroxyl group of T432 that is therefore phosphorylated before S431 [13,16]. The dephosphorylation reaction then proceeds in the same order (pT432 is followed by pS431), such that the overall process from the hypo-phosphorylated to the hyper-phosphorylated and back to the hypo-phosphorylated state of KaiC involves the following stages: TS → pTS → pTpS → TpS → TS [11,12]. The phosphorylation status of KaiC is therefore a marker for the phase of the *in vitro* oscillator [12].

KaiA stimulates KaiC phosphorylation that 'ratchets' the clockwork

KaiC can auto-phosphorylate, especially at low temperatures (e.g., 4 °C). At physiological temperatures, however, KaiA greatly stimulates the autokinase activity of KaiC [15,18]. Specifically, binding of KaiA homo-dimer to a KaiC C-terminal tail or 'tentacle' activates the kinase in the CII ring [19,20], thus triggering phosphorylation of T432 and S431 residues (Figures 2 and 3). Phosphorylation of T432 and S431/T426 leads to increased molecular