



## Biochemistry That Times the Day

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“Timing is everything” appears to be a motto not limited to human existence. In fact, keeping track of time is so important that organisms ranging from algae and insects to mammals rely on circadian clocks, endogenous molecular oscillators with a period of approximately 24 h, to regulate diverse processes that include the cell cycle, gene expression, and metabolism. Although all biological clocks are characterized by three diagnostic properties, (i) an internal timer ticking with an ~24 h period, (ii) entrainment of the endogenous timer by exogenous cues such as light, and (iii) temperature compensation, the molecular players constituting the clock are not conserved across all organisms. Beyond similarities and differences as far as the cogs and gears that assemble the clock are concerned, recent research has brought to light the increasingly complex nature of clock mechanism and regulation. Thus, the assumption that a so-called transcription/translation-based feedback loop (TTFL) is at the heart of all biological clocks appears to be an oversimplification at best; in all systems, a post-translational component of the timing circuitry is becoming apparent. This issue of *Biochemistry* bears witness to the advances in clock research achieved over the past decade by presenting a collection of seven Current Topics articles (DOIs 10.1021/bi5007354, 10.1021/bi500707c, 10.1021/bi500731f, 10.1021/bi5005624, 10.1021/bi500922q, 10.1021/bi501089x, and 10.1021/bi5008386). These papers penned by a diverse group of clock researchers attest to the universal importance of circadian timing and provide insight into progress in regard to clock structure, function, and mechanism in all walks of life, from seasonal flowering to circadian rhythmicity in carcinogenesis and chemotherapy.

### ■ MOLECULAR BASES AND MECHANISMS OF CIRCADIAN CLOCKS

Clock genes giving rise to circadian rhythms<sup>1</sup> have been identified in diverse model organisms, including mammals,<sup>2</sup> the fungus *Neurospora crassa*,<sup>3</sup> the fruit fly *Drosophila melanogaster*,<sup>4</sup> a small flowering plant from the mustard family, *Arabidopsis thaliana*,<sup>5</sup> and the cyanobacterium *Synechococcus elongatus*.<sup>6</sup> Key rhythms generated in all these systems concern oscillations in the transcripts and/or proteins of particular clock genes and are sustained by autoregulatory feedback loops.<sup>7,8</sup> Examples are *mPer1*, *mPer2*, and *mPer3* as well as *cry1*, *cry2*, and *Bmal1* in mammals, *clk*, *per*, and *tim* in the fly, *frq* and WC-1 in *Neurospora*, and *kaiA* and *kaiBC* in the cyanobacteria. The core clock mechanism in the mammalian clock entails the transcription factors CLOCK and BMAL1 that form a heterodimer to activate transcription of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes (DOI: 10.1021/bi500707c). The negative arm of this TTFL is comprised of the PER and CRY proteins that heterodimerize and interact with

CLOCK:BMAL1 after translocating back to the nucleus to inhibit their own transcription. The robustness of this primary TTFL is enhanced by a secondary feedback loop that involves nuclear hormone receptors *Rev-erbs* and *RORs* that act as negative and positive regulators, respectively, of BMAL1 transcription. By contrast, a very different set of proteins forms the circadian timer in the simplest organisms to possess a clock, the cyanobacteria, e.g., *S. elongatus*.<sup>9,10</sup> The fact that the KaiA and KaiC proteins positively and negatively regulate *kaiBC* transcription, respectively,<sup>6</sup> is consistent with a TTFL model. However, the subsequent findings that the cyanobacterial clock ticks without *de novo* synthesis of clock gene mRNAs<sup>11</sup> and that the clock can be reconstituted *in vitro* from the KaiA, KaiB, and KaiC proteins in the presence of ATP<sup>12</sup> point to a nontranscriptional oscillator (NTO) (DOI: 10.1021/bi501089x) as the driving force of circadian rhythm in these organisms. Indeed, it was shown that this KaiABC post-translational oscillator (PTO) represents the master timer, with the TTFL and clock-controlled gene expression being under its control.<sup>13,14</sup> No evolutionary relationship appears to exist between the molecular players in the KaiABC PTO and the CLOCK/BMAL1-driven master TTFL in mammals. Therefore, clocks may have arisen by evolution on Earth more than once, endowing organisms with a powerful tool to anticipate periodic environmental changes and an adequate response at their disposal. The contribution in this Current Topic collection on clocks in algae by Noordally and Millar discusses the gating of cell division by the circadian clock in *Chlamydomonas reinhardtii* and its initiation coinciding with the beginning of the subjective night (DOI: 10.1021/bi501089x). Thus, *C. reinhardtii* may minimize the potentially detrimental effects of UV radiation on nuclear DNA replication.<sup>15</sup> This observation provides support for the “escape from light” hypothesis that postulated an early adaptive advantage for organisms by shifting cellular processes sensitive to light into the subjective night phase.<sup>16</sup>

### ■ REGULATION BY AND OF THE MAMMALIAN CLOCK

Two reviews in the present Current Topics collection are concerned with clock regulation. Sancar and co-workers discuss how the mammalian clock interfaces with other regulatory systems (DOI: 10.1021/bi5007354), and Kojima and Green provide an update on post-transcriptional regulation of the clock in mammals (DOI: 10.1021/bi500707c). As covered in detail in the first contribution, the integration of the circadian clock into the entire transcriptional and signal transduction

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networks might result in the assumption that the clock plays a key role in the main intracellular communication systems and is of critical importance for physiology and pathophysiology. Indeed, the relationship between clock and sleep, physical and mental performance, or the onset of allergic reactions and cardiovascular episodes is well-established by comparison.<sup>17–20</sup> However, mice with mutations in key clock components such as *Clock*, *Cry*, or *Per* exhibit essentially normal growth and life spans.<sup>21</sup> Further, the contributions of the molecular clock via cell cycle and DNA damage check points to normal cell physiology appear to be nonessential, and clock disruption and cancer have not been causally linked (DOI: 10.1021/bi5007354). Instead, it appears that interruption of normal clock rhythm may obstruct cancer growth.<sup>22</sup> As far as the role of the clock in DNA repair pathways is concerned, it has been established that the rate of nucleotide excision repair exhibits a circadian dependence,<sup>23–25</sup> an observation that could be exploited for the development of optimally timed chemotherapies and radiation therapies (chronotherapy).

Key benefits of post-transcriptional regulatory mechanisms are the added flexibility to overall gene expression and rapid alteration of protein levels without the need for *de novo* transcript synthesis (DOI: 10.1021/bi500707c). Although circadian rhythmicity is a hallmark of cellular temporal organization and transcriptional control is central to the circadian clock (TTFL), expression of clock-controlled genes (ccgs) does not entail pervasive rhythmicity at the mRNA and protein levels. Thus, as many as half of the proteins that are rhythmically expressed appear not to display rhythmicity at the level of their transcript.<sup>26,27</sup> Moreover, it was recently shown that the mRNAs of as many as 30% of ccgs are post-transcriptionally regulated by microRNAs (miRNAs), perhaps controlling the amplitude and/or phase of rhythmic expression patterns.<sup>28</sup> Circadian proteomics brought to light the fact that expression of approximately 5–10% of proteins in mouse liver is rhythmic,<sup>27,29</sup> an amount quite similar to the amount of ccgs identified by genomic analyses. A key concern in the identification of ccgs is the role of normalization;<sup>30</sup> i.e., at what point is the amplitude of a rhythm too weak to be considered significant in terms of the modulation of physiological output? Another open question with regard to the control of circadian output pathways concerns the relative importance of ccgs and rhythmically expressed proteins as the regulating entities (DOI: 10.1021/bi500707c). What appears clear from genomic, proteomic, and metabolomic analyses is that transcriptional mechanisms alone are insufficient to sustain all of the rhythmic mRNA expression and that the regulatory network involves multiple layers.

## ■ CLOCK ARCHITECTURE

A full understanding of clock function and mechanism requires detailed structural information about the main transcriptional regulators and the interactions between them. The cyanobacterial clock system was the pioneer in terms of the determination of three-dimensional (3D) structures of clock proteins (for cyanobacteria, KaiA, KaiB, and KaiC) and the application of that knowledge toward understanding how the clock ticks.<sup>10,13,14,43,45</sup> As summarized by Gustafson and Partch, major progress in other systems toward the determinations of high-resolution structures of clock proteins and their complexes has been made in the past couple of years (DOI: 10.1021/bi500731f). In particular, these concern crystal structures of the CLOCK:BMAL1 heterodimer from mouse (mCLOCK:mB-

MAL1), encompassing the basic helix–loop–helix (bHLH) and tandem PER-ARNT-SIM A and B (PAS-AB) domains, and the heterodimer formed by their bHLH domains in complex with the cognate E-box recognition sequence. Further crystal structures include PAS-AB portions of mPER1, mPER2, and mPER3, the photolyase homology regions along with the conserved CC helix motif (PHR-CC) of mCRY1 and mCRY2, and ligand binding domains (LBDs) of human REV-ERB $\alpha$ , REV-ERB $\beta$ , ROR $\alpha$ , ROR $\beta$  (from rat), and ROR $\gamma$  with a variety of compounds bound. For a more detailed description of the structures, please consult DOI: 10.1021/bi500731f. From the available data, it is evident that clock proteins forming the main TTFLs share structural motifs that allow interactions between them or with DNA. Examples of the former are tandem PAS domains in CLOCK and BMAL1 that mediate formation of the heterodimer<sup>31</sup> and in PER proteins, where they control formation of both homo- and heterodimers.<sup>32,33</sup> Another concern is the shared nuclear receptor architecture of the REV-ERB and ROR proteins that use their LBDs to bind different ligands.<sup>34</sup> Cryptochromes do not share architectural features with CLOCK, BMAL1, and PER proteins because they lack PAS domains but instead contain the PHR that is structurally similar to photolyase.<sup>35</sup> However, unlike the latter, they possess a disordered C-terminal region, a feature that they share with CLOCK and BMAL1 (where it serves the regulation of their activity)<sup>2,36–38</sup> as well as PER (where it contains binding sites for kinases and CRY).<sup>39,40</sup> The presence of such intrinsically disordered domains (IDDs)<sup>41</sup> is not just a shared property among mammalian clock proteins and other components involved in transcriptional regulation;<sup>42</sup> disordered C-terminal regions also figure prominently in the proteins that assemble the cyanobacterial KaiABC PTO.<sup>10,43</sup> A structural and functional understanding of these regions, e.g., the C-terminal tail of KaiB that is important for proper rhythmicity of the cyanobacterial timer,<sup>44</sup> has remained elusive thus far, despite the use of a hybrid structural biology approach to dissect the KaiABC clock.<sup>45</sup> The challenges posed by this “simple clock”, regardless of the application of a battery of structural and molecular biology as well as genetic tools, may offer a lesson for the path ahead in structural studies directed at the cogs and gears of the arguably more complex mammalian clock (DOI: 10.1021/bi500731f). A key aspect of the latter system where insights from structural biology are currently lacking concerns the interactions between cryptochromes and CLOCK:BMAL1.

## ■ CONVERGENT CLOCK MODELS IN LOWER AND HIGHER EUKARYOTES: HOW FUNGI TELL TIME

*N. crassa* has served as an outstanding model organism for studies of the clock, thanks to the similar organization of its circadian oscillator relative to those of higher eukaryotes despite the evolutionary distance.<sup>46</sup> Thus, WHITE COLLAR transcription factors WC-1 and WC-2 correspond to CLOCK and BMAL1 and form the WHITE COLLAR complex (WCC)<sup>47</sup> that activates the transcription of the *frequency* (*frq*) gene. FREQUENCY (FRQ) together with the FRQ-interacting RNA helicase (FRH) then function as the negative elements in the core feedback loop of the *Neurospora* clock.<sup>48,49</sup> Liu and colleagues provide a *frq*/FRQ-centric view of the *Neurospora* oscillator and highlight mechanisms operating at the transcriptional, post-transcriptional, co-translational, and post-translational levels (DOI: 10.1021/bi5005624). Transcriptional regulation of *frq* proceeds via phosphorylation of WCC that inhibits the activator. Several sites are affected by kinase

activity, whereby phosphorylation by protein kinase A occurs in a fashion that is independent of FRQ, followed by further phosphorylation events by casein kinases that are recruited by FRQ.<sup>50,51</sup>

Phosphorylation is ubiquitous in clock systems and critical to negative feedback via a multitude of mechanisms that include localization, inhibition of protein–protein interactions, and/or activity. Thus, phosphorylation is also of central importance in the cyanobacterial KaiABC oscillator, where the alternating phosphorylation and dephosphorylation of KaiC in the PTO determine period, phase, and the composition of heteromultimeric complexes among the three Kai proteins.<sup>10,14</sup> Beyond the more familiar regulation of oscillators at the transcriptional and post-translational levels, the results of a recent investigation suggest that *frq* codon usage affects FRQ expression and function.<sup>52</sup> Both the *frq* clock gene from *Neurospora* and *kaiBC* in cyanobacteria<sup>53</sup> contain nonoptimal codons in their open reading frames. When these were optimized in the N-terminal region, FRQ expression levels increased but the clock function was impaired, indicating the presence of yet another layer of control in the complex network of clock regulation and mechanism (DOI: 10.1021/bi5005624). On the other hand, when the *kaiBC* sequences were optimized in cyanobacteria, clock function appeared to be enhanced, but growth of cells under poor environmental conditions (low temperature) was compromised.<sup>53</sup>

## ■ A CLOCK FOR ALL SEASONS: FLOWERING AND PHOTOPERIOD

The function of the circadian clock is commonly associated with the diurnal cycle but perhaps less with physiological and developmental events that occur throughout the year. Shim and Imaizumi in their Current Topics review shed light on the molecular mechanisms of the clock in *Arabidopsis* that underlie seasonal changes (DOI: 10.1021/bi500922q). Plants exhibit many circadian rhythms that include photosynthesis, photorespiration, stomatal opening, and movement of leaves, but the clock also controls changes that occur during the year such as flowering and dormancy.<sup>54,55</sup> The photoperiod or day length can serve as a signal of seasonal change for plants by way of photoreceptors that sense the surrounding light environment and inform clock-controlled transcription. Just like the clocks of higher organisms, the regulation of the *Arabidopsis* clock features positive and negative feedback loops, phosphorylation of clock components by casein kinase, and proteolytic degradation.<sup>56</sup> The photoperiodic flowering response involves the FLOWERING LOCUS T (FT) protein that accelerates flowering.<sup>57</sup> FT induction is regulated by the CONSTANS (CO) transcriptional activator, whereby the daily expression patterns of CO are governed at the level of transcription by the circadian clock-regulated FKF1, GI, and CDF proteins. CO protein is also regulated at the post-translational level; direct and indirect interactions with photoreceptors FKF1, CRY1, and CRY2 affect CO stability, and light therefore determines the timing of CO accumulation and its induction of FT.<sup>58</sup>

Beyond the advances in the characterization of positive and negative regulators of feedback loops in the *Arabidopsis* circadian clock and the mechanism of the photoperiodic response, recent research has also uncovered additional layers of post-transcriptional and -translational clock regulation (DOI: 10.1021/bi5005624). For example, phosphorylation of CCA1 protein and alternative splicing of CCA1 transcripts appear to play a role in the temperature compensation of the circadian

oscillator.<sup>59,60</sup> Other effects of the photoperiod concern reactive oxygen species (ROS) homeostasis; there is mounting evidence that the circadian clock is involved in the regulation of cellular processes that control ROS physiological levels, e.g., via the peroxiredoxin proteins (PRXs) (DOI: 10.1021/bi5005624). In *Arabidopsis*, CCA1 appears to be involved in maintaining the diurnal oscillation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels.<sup>61</sup> The clock-dependent regulation of the cellular ROS level, e.g., those of H<sub>2</sub>O<sub>2</sub>, is clearly important for plant development (leaf growth) and defense responses, although the mechanisms ensuring the timing of ROS signals remain to be worked out.

## ■ NOT SO SIMPLE BEGINNINGS: ALGAE GOT RHYTHM

Studies of circadian rhythms in algae, a large and diverse group of eukaryotic organisms that include uni- and multicellular forms, have contributed significantly to our understanding of molecular clocks since their beginnings more than six decades ago.<sup>62</sup> Noordally and Millar discuss the results of research into circadian rhythms from seven algal species and review the integration of genomic, transcriptomic, metabolomic, and proteomic data using computational and mathematical approaches (DOI: 10.1021/bi501089x). Early insights into clock mechanism came from investigations of the circadian rhythm of photosynthesis in *Acetabularia* that persisted in the absence of nuclear transcription and are therefore challenging the dogma of TTFLs as underlying all molecular timers. Such NTOs have now been found in mammals, insects, fungi, cyanobacteria, the alga *Ostreococcus tauri*, and red blood cells (DOI: 10.1021/bi501089x, DOI: 10.1021/bi5008386, and ref 63). Comparison between rhythms in wild-type and mutant *C. reinhardtii* in space and on earth demonstrated no changes as a result of the different environments, confirming the existence of endogenous circadian behavior in the absence of external cues.<sup>64</sup> Another comparative analysis of clocks from eukaryotes established a smaller degree of conservation among protein components constituting the TTFLs compared to that of kinases and phosphatases participating in circadian oscillators, thereby providing support for phosphorylation-based signaling as a central element in the ancestral clock.<sup>65</sup> Thus, the function of *O. tauri* CK1 appears to be very similar to that of CK1  $\epsilon$  in the human clock (DOI: 10.1021/bi501089x). Protein kinases probably play a role in NTOs as well, as indicated by the distorted PRX rhythm caused by an inhibitor of casein kinase 1 (CK1).<sup>65</sup> One of the challenges concerning the roles of kinases is the identification of targets and their partitioning between the canonical TTFL and NTO mechanisms. Algal systems such as *O. tauri* and *C. reinhardtii* constitute valuable model organisms for systems biology and genetic investigations of the clock and ecological and physiological studies thanks to rapid culturing and ready genetic manipulation as well as their relevance in terms of mechanism and regulatory pathways for the human oscillator.

## ■ PERSPECTIVE: PTOS AND TTFLS

The pre-eminent example of “biochemistry that times the day” is the case of the cyanobacterial KaiABC system<sup>6</sup> that includes a biochemical timing mechanism that is strictly post-translational<sup>11</sup> and can be reconstituted *in vitro*.<sup>12</sup> This is the system on which we, the organizers of this Topics Collection, work, but we chose not to include a separate contribution because the cyanobacterial clockwork has been reviewed extensively and



recently.<sup>10,66</sup> As mentioned above, the cyanobacterial clock system has been the pioneer in applying 3D structural information to understanding clock mechanisms;<sup>10,13,14,43,45</sup> however, an underappreciated aspect of the cyanobacterial clock is that the PTO operates within a larger TTFL, and in fact, the first hypotheses of clock mechanism in cyanobacteria borrowed the conceptual basis of TTFLs from eukaryotes.<sup>6</sup> Later, the dispensability of the TTFL became obvious,<sup>11</sup> which elicited a soul-searching process in which an *in vitro* oscillator that is thought to act as a PTO “quartz crystal” *in vivo* was identified.<sup>12,13</sup> Therefore, the larger cyanobacterial system comprises a coupled PTO/TTFL circuitry, and these coupled oscillators promote robustness and resilience.<sup>13,66,67</sup>

Could it be that a PTO underlies the TTFL in eukaryotes, as well? Despite the enormous progress made on the elucidation of the eukaryotic clockworks, there have been recent attempts in the field of chronobiology to re-evaluate the central role of a TTFL in these circadian pacemakers.<sup>68</sup> For example, it is noteworthy that key circadian properties, among them the 24 h period and temperature compensation, are difficult to explain by the current TTFL model (first, this TTFL could just as easily be a 3–4 h oscillator, and second, transcription and translation are intrinsically temperature-dependent). Undoubtedly, the paradigm-shifting discovery of an *in vitro* 24 h oscillator in cyanobacteria inspired eukaryotic clock biologists to think “outside the (TTFL) box”, resulting in the unexpected revelation of NTOs/PTOs in mammals and other eukaryotes (DOI: 10.1021/bi5008386).<sup>63,65,69</sup> The relationship between the PRX NTO/PTO and the TTFL that drives circadian transcription cycles is presently unclear; at least in cyanobacteria, the PRX cycles appear to be uncoupled from both the KaiABC PTO and the larger TTFL.<sup>69</sup>

It may be opportune at this crossroads in chronobiological research to consider alternatives for the eukaryotic pacemaker such as a core PTO embedded in a TTFL akin to that found in cyanobacteria. What are the potential benefits of a biochemical (PTO) oscillator embedded within a genetic (TTFL) oscillator? A core oscillator that is composed of biochemical reactions among thousands of molecules per cell should be more robust in the face of metabolic noise than one founded on transcriptional activity. This is particularly true for cells that must maintain precise timekeeping during cell division, when the ratio of DNA to transcriptional factors can change during replication and when DNA can become less accessible as chromatin structure changes in preparation for division. The advantage provided by a biochemical oscillator such as the KaiABC system in cyanobacteria is that a post-translational system could be less susceptible to the influences of cell division or major changes in metabolic rate than one based on transcriptional and translational rates.<sup>68</sup>

A circadian mechanism that can maintain its accuracy in the face of turbulent metabolic changes caused by cell division and environmental stresses could have provided an evolutionary driving force for convergent circadian clock mechanisms among diverse organisms. Discussions of eukaryotic circadian mechanisms frequently identify the TTFL as providing essential feedback, but the TTFL itself cannot explain the 24 h time constant. Therefore, an amorphous “delay” is often introduced to justify how eukaryotic clocks can have such a long period, and the phosphorylation rate of clock proteins has been proposed as a possible candidate to provide this temperature-compensated delay. Perhaps in conjunction with the dramatic changes in subcellular concentrations caused by nuclear

translocation of central clock proteins, a strictly biochemical timer provides a temperature-compensated segment of the circadian cycle and partially establishes the long time constant of the clock. Perhaps in eukaryotes, this biochemical timer is not an oscillator, but a temperature-compensated “hourglass” timer. Whichever alternative is vindicated by future research, a discussion of “biochemistry that times the day” is not only relevant to the cyanobacterial *in vitro* oscillator but also potentially critical for understanding circadian timing in all organisms. Hence, the relevance of this collection of Current Topics in circadian clocks.

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### Notes

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