Robust circadian clocks from coupled protein-modification and transcription–translation cycles

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The cyanobacterium *Synechococcus elongatus* uses both a protein phosphorylation cycle and a transcription–translation cycle to generate circadian rhythms that are highly robust against biochemical noise. We use stochastic simulations to analyze how these cycles interact to generate stable rhythms in growing, dividing cells. We find that a protein phosphorylation cycle by itself is robust when protein turnover is low. For high decay or dilution rates (and compensating synthesis rates), however, the phosphorylation-based oscillator loses its integrity. Circadian rhythms thus cannot be generated with a phosphorylation cycle alone when the growth rate, and consequently the rate of protein dilution, is high enough; in practice, a purely posttranslational clock ceases to function well when the cell doubling time drops below the 24-h clock period. At higher growth rates, a transcription–translation cycle becomes essential for generating robust circadian rhythms. Interestingly, although a transcription–translation cycle is necessary to sustain a phosphorylation cycle at high growth rates, a phosphorylation cycle can dramatically enhance the robustness of a transcription–translation cycle at lower protein decay or dilution rates. In fact, the full oscillator built from these two tightly intertwined cycles far outperforms not just each of its two components individually, but also a hypothetical system in which the two parts are coupled as in textbook models of coupled phase oscillators. Our analysis thus predicts that both cycles are required to generate robust circadian rhythms over the full range of growth conditions.

Kai | oscillations

Many organisms use circadian clocks to anticipate changes between day and night (1). It had long been believed that these clocks are driven primarily by transcription–translation cycles built on negative feedback. However, although some circadian clocks can maintain robust rhythms for years in the absence of any daily cue (1), recent experiments have vividly demonstrated that gene expression is often highly stochastic (2). This raises the question of how these clocks can be so robust against biochemical noise. In multicellular organisms, the robustness might be explained by intercellular interactions (3, 4), but it is now known that even unicellular organisms can have very stable circadian rhythms. The clock of the cyanobacterium *Synechococcus elongatus*, for example, has a correlation time of several months (5), even though the clocks of the different cells in a population hardly interact with one another (5, 6). How circadian clocks can be so stable even at the single cell level is not understood.

Interestingly, it has recently been discovered that the *S. elongatus* clock combines a transcription–translation cycle (TTC) with a protein phosphorylation cycle (PPC). The central components of the clock are the three genes *kaiA*, *kaiB*, and *kaiC* (7). Under continuous light conditions, the levels of mRNA from the *kaiBC* operon and of the protein KaiC oscillate in a circadian fashion (8); moreover, overexpression of KaiC abolishes *kaiBC* expression (9, 10). These observations led to the proposal that the Kai system is a transcription–translation oscillator, with KaiC negatively regulating its own transcription. In 2005, however, Kondo and coworkers showed that KaiC, which is a hexamer with

two phosphorylation sites per monomer (11), is phosphorylated in a cyclical manner with a period of 24 h, even when *kaiBC* transcription is inhibited (12). Still more remarkably, the rhythmic phosphorylation of KaiC could be reconstituted in the test tube in the presence of only KaiA, KaiB, and ATP (13). This raised the possibility that the principal pacemaker of the clock is not a TTC, but a PPC (13). Yet, in 2008, the same group showed that circadian oscillations of gene expression persist even when KaiC is always held in a highly phosphorylated state (14). They thus concluded that the clock is driven by both a TTC and a PPC and suggested that the interactions between the two oscillators may enhance the robustness of the clock (14).

Here, we use mathematical modeling to study how a protein-modification oscillator and a transcription–translation oscillator interact in growing, dividing cells. To this end, we study four models, schematically shown in Fig. 1: (*i*) *PPC-in vitro* model (Fig. 1*B*); (*ii*) *PPC-in vivo* model (Fig. 1*C*); (*iii*) *PPC-TTC* model (Fig. 1*A*); (*iv*) *TTC-only* model (Fig. 1*D*). We first study the *PPC-in vitro* model, which describes the PPC that has been reconstituted in the test tube (13) (Fig. 1*B*). In this system, the total number of each Kai protein is *constant*—they are neither produced nor destroyed—and only the PPC is operative. We show that in this case the PPC is highly robust against noise arising from the intrinsic stochasticity of chemical reactions. Even for reaction volumes smaller than the typical volume of a cyanobacterium, the correlation time is longer than that observed experimentally (5). Living cells, however, constantly grow and divide, and proteins must thus be synthesized to balance dilution. In fact, dilution can be thought of as introducing an effective protein degradation rate set by the cell doubling time. We therefore next study the *PPC-in vivo* model (Fig. 1*C*), which describes a PPC in which the Kai proteins are produced and degraded with rates that are constant in time. The simulations reveal that protein synthesis and decay dramatically reduce the viability of the PPC; we predict that for a cell doubling time of 24 h and a bacterial volume of 1 μm³, the PPC dephases in roughly 10 days, much faster than real *S. elongatus* (5). This is because the constant synthesis of proteins, which we assume are all initially created in the same phosphorylation state, necessarily injects KaiC with the "wrong" phosphorylation level at certain phases of the cycle (1, 15, 16); if these appear fast enough, they can destroy the oscillation. One role of the TTC is thus to introduce proteins only when the phosphorylation state of the freshly made KaiC matches that of the PPC. Our simulations of the *PPC-TTC* model (Fig. 1*A*), which combines a PPC and a TTC, reveal that a TTC can indeed greatly
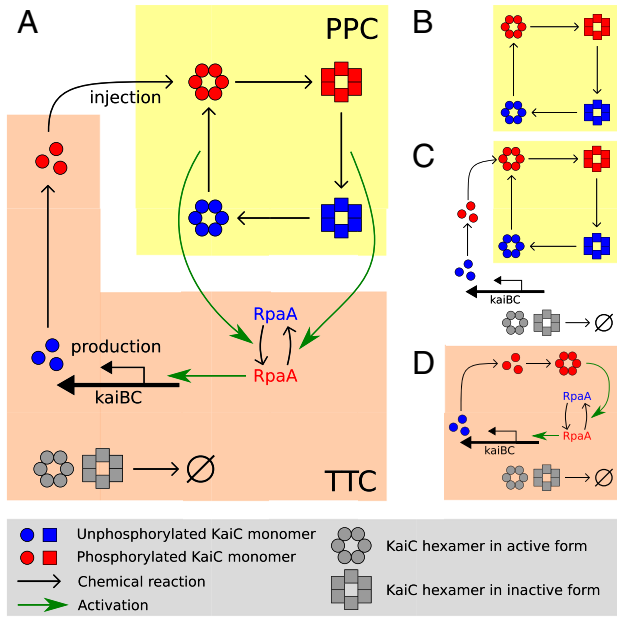

Fig. 1. Overview of the models studied in this manuscript. (*A*) *PPC-TTC* model: A TTC of *kaiBC* expression (orange background) interacts with a KaiC PPC (yellow background) (40). KaiC is a hexamer that, in our model, switches between an active conformational state (circles) in which its phosphorylation level tends to rise and an inactive state (squares) in which it tends to fall. Active KaiC activates RpaA, whereas inactive KaiC inactivates RpaA; active RpaA (red) activates *kaiBC* expression, leading (after a delay) to the injection of fully phosphorylated KaiC into the PPC. (*B*) *PPC-in vitro* model: Only the PPC is present, and the concentration of each Kai protein is constant. (*C*) *PPC-in vivo* model: Only the PPC is present, but all Kai proteins are now continually synthesized and degraded, with rates that are constant in time. (*D*) *TTC-only* model: Only the TTC is present, and KaiC is always in a highly phosphorylated state. *RpaA* and *kaiA* are expressed constitutively; roles of KaiA and KaiB are discussed in the text.

enhance the robustness of the PPC, yielding correlation times consistent with those measured experimentally (5). Finally, we consider whether the PPC is needed at all, or whether one could build an equally good circadian clock using only a TTC; to this end, we study the *TTC-only* model (Fig. 1*D*). We find that it is possible to construct a TTC with a period of 24 h and the observed correlation time of a few months (5). However, this comes at the expense of very high protein synthesis and decay rates, which impose an extra energetic burden on the cell. Our results thus suggest that a PPC allows for a more robust oscillator at a lower cost. Although our models are simplified, we argue in *Principal Pacemaker* and *Discussion* that our qualitative results are unavoidable consequences of the interaction between a circadian clock and cell growth and so should hold far more generally.

Results

A. *PPC-in Vitro*: A Protein Phosphorylation Cycle with Constant Protein Concentrations Is Highly Robust. The PPC has been characterized in detail (Fig. 1*B*). KaiC forms a hexamer (11) with two phosphorylation sites per protein monomer, which are phosphorylated and dephosphorylated in a definite sequence as a result of KaiC's autokinase and autophosphatase activity (17, 18). KaiA stimulates KaiC phosphorylation (19, 20), whereas KaiB negates the effect of KaiA (19–22). Thanks to the wealth of available experimental data, the PPC has proven a fruitful system for mathematical modeling (18, 23–31) (for a review, see ref. 32).

In this manuscript, we adopt the mathematical model developed by us (26). In this model, each KaiC hexamer has an intrinsic ability to cyclically phosphorylate and dephosphorylate itself, while the phosphorylation cycles of the individual hexamers are synchronized via a mechanism that we called "differential af-

finity" (18, 23, 26, 27): KaiA stimulates KaiC phosphorylation, but the limited supply of KaiA dimers binds preferentially to those KaiC molecules that are falling behind in the cycle, allowing them to catch up. Specifically, in our model each KaiC hexamer can switch between an active conformational state C_i, where the number i of phosphorylated monomers tends to increase, and an inactive state $\tilde{C}_i$, where i tends to decrease (Fig. 1*B*); KaiA stimulates phosphorylation of active KaiC, but is sequestered by complexes containing KaiB and inactive KaiC. KaiC in the inactive state can thus delay the progress of fully dephosphorylated hexamers that have already switched back to the active state and are ready to be phosphorylated again. With A and B denoting, respectively, a KaiA dimer and a KaiB dimer, the model becomes

$$C_i \underset{b_i}{\overset{f_i}{\rightleftarrows}} \tilde{C}_i, \qquad C_i + A \underset{k_i^{\text{Ab}}}{\overset{k_i^{\text{Af}}}{\rightleftarrows}} AC_i \overset{k_{\text{pf}}}{\rightarrow} C_{i+1} + A, \qquad \textbf{[1]}$$

$$\tilde{C}_i + B \underset{\tilde{k}_i^{\text{Bb}}}{\overset{2\tilde{k}_i^{\text{Bf}}}{\rightleftarrows}} B\tilde{C}_i, \qquad B\tilde{C}_i + B \underset{2\tilde{k}_i^{\text{Bb}}}{\overset{\tilde{k}_i^{\text{Bf}}}{\rightleftarrows}} B_2\tilde{C}_i, \qquad \textbf{[2]}$$

$$B_x\tilde{C}_i + A \underset{\tilde{k}_i^{\text{Ab}}}{\overset{x\tilde{k}_i^{\text{Af}}}{\rightleftarrows}} AB_x\tilde{C}_i, \qquad AB_2\tilde{C}_i + A \underset{2\tilde{k}_i^{\text{Ab}}}{\overset{\tilde{k}_i^{\text{Af}}}{\rightleftarrows}} A_2B_2\tilde{C}_i, \qquad \textbf{[3]}$$

$$C_i \underset{k_{\text{dps}}}{\overset{k_{\text{ps}}}{\rightleftarrows}} C_{i+1}, \qquad \tilde{C}_i \underset{\tilde{k}_{\text{dps}}}{\overset{\tilde{k}_{\text{ps}}}{\rightleftarrows}} \tilde{C}_{i+1}, \qquad \textbf{[4]}$$

$$B_x\tilde{C}_i \underset{\tilde{k}_{\text{dps}}}{\overset{\tilde{k}_{\text{ps}}}{\rightleftarrows}} B_x\tilde{C}_{i+1}, \qquad A_yB_x\tilde{C}_i \underset{\tilde{k}_{\text{dps}}}{\overset{\tilde{k}_{\text{ps}}}{\rightleftarrows}} A_yB_x\tilde{C}_{i+1}. \qquad \textbf{[5]}$$

This model reproduces the phosphorylation behavior of KaiC in vitro not only when all Kai proteins are present, but also when KaiA and/or KaiB are absent (26). It moreover correctly predicted the experimentally observed disappearance of oscillations when the KaiA concentration is raised (18, 33), a success that strongly supports the idea that KaiA sequestration is the primary driver of synchronization. Our model does not feature monomer exchange between KaiC hexamers, an alternative means of synchronization (24) that has been observed in experiments (28, 34); we and others find that monomer exchange is not critical for stable oscillations (18, 26, 27). In *SI Text*, we show that similar results are obtained with a model that focuses on the phosphorylation cycle of individual KaiC monomers (17, 18) rather than of KaiC hexamers.

We quantify our model's robustness to chemical noise by performing Monte Carlo simulations of the chemical master equation (35) describing the mass-action kinetics associated with reactions **1**–**5** in a well-mixed container. In our simulations, we vary the reaction volume V but adjust the protein copy numbers so that the concentrations of the Kai proteins remain constant, at levels comparable to those used in vitro (33, 34). Fig. 2 shows as a function of volume the correlation number of cycles $n_{1/2}$, defined as the number of cycles after which the standard deviation in the phase of the oscillation is half a day (31). One issue that arises in comparing our results to the measured in vivo clock robustness is that the Kai proteins appear to be present in living cells in a ratio at which the in vitro system would not oscillate (21, 33, 34). It has been suggested that this discrepancy may indicate that the clock reactions are confined to a subdomain of the cell from which some KaiB and KaiC molecules are excluded (21), allowing the reactions to proceed at roughly the in vitro ratio (see *SI Text*). If we take the volume of this domain to be $V \sim 1 \ \mu\text{m}^3$, comparable to the size of the entire cell, then Fig. 2 shows that $n_{1/2} \approx 200$, consistent with the measured 166 ± 100 (5). Even for $V \sim 0.5 \ \mu\text{m}^3$, we find that $n_{1/2} \approx 102$, still within the experimental bounds (in contrast to predictions of some alternative
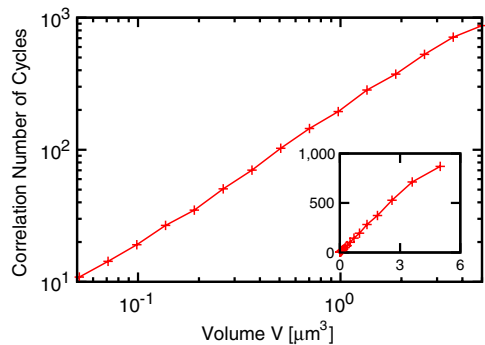
Fig. 2. *PPC-in vitro* model (Fig. 1*B*). Correlation number of cycles, $n_{1/2}$, as a function of the reaction volume V. For $V \approx 1 \ \mu m^3$, comparable to the size of a cyanobacterium, $n_{1/2} \approx 200$, in agreement with experiment (5). (*Inset*) The same data on a linear scale. The protein concentrations are those used in the in vitro experiments (33, 34): $[A]_T = 0.58 \ \mu M$; $[B]_T = 1.75 \ \mu M$; $[C]_T = 0.58 \ \mu M$. For other parameters, see Table S1. For a time trace of the phosphorylation level $p(t)$, see Fig. S1.

models (18, 31); see *SI Text*). Our model thus predicts that the PPC is resistant to noise arising from the stochastic nature of chemical reactions.

B. *PPC-in Vivo*: A Phosphorylation Cycle with Constant Protein Synthesis and Degradation Rates Is not Stable. Fig. 2 shows that the PPC is highly robust when the total concentrations of the Kai proteins are strictly constant. But, in vivo, proteins are continually being synthesized and degraded. To study how this affects the PPC, we consider the *PPC-in vivo* model (Fig. 1*C*). In this model, the Kai proteins are produced and degraded in a stochastic (memoryless) fashion with rates that are constant in time, with the effects of active degradation (36) and of passive dilution lumped into a single first-order decay rate μ (see *SI Text*).

Fig. 3 shows the performance of the PPC as a function of degradation rate and cell volume; here, and in the models below, the synthesis rates are adjusted so that the mean concentrations are constant and equal to those used in the previous section. The PPC's robustness clearly decreases dramatically with increasing protein synthesis and decay rate. For a volume comparable to that of a cyanobacterium and a degradation rate of 0.03 h^{-1}, the correlation time is less than 20 days, much lower than that observed in vivo (5). This degradation rate is precisely the effective rate arising from protein dilution with a cell doubling time of 24 h. It is known, however, that KaiC is also degraded actively at a rate as high as 0.1 h^{-1} (36), leading to still worse stability.
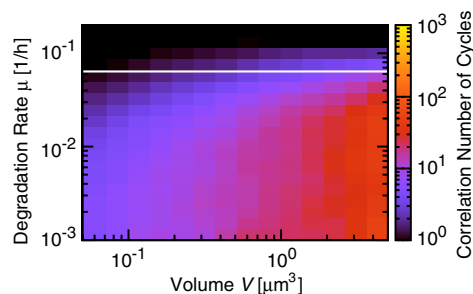

Fig. 3. *PPC-in vivo* model (Fig. 1*C*). Correlation number of cycles, $n_{1/2}$, as a function of reaction volume V and degradation rate μ. $n_{1/2}$ decreases as μ increases. Thus, a system in which the Kai proteins are produced and degraded with constant rates cannot sustain a stable phosphorylation cycle when the growth rate is high. The bifurcation line $\mu = 0.0621$ h^{-1}, where the system undergoes a supercritical Hopf bifurcation in the deterministic limit, is shown in white. KaiA, KaiB, and KaiC are produced with rates such that the average total concentrations equal those used in the in vitro experiments (see Fig. 2). For a time trace of $p(t)$, see Fig. S1.

The disappearance of the oscillations for higher protein synthesis and decay rates can be understood by noting that fresh KaiC hexamers are made in a fixed phosphorylation state, which then has to catch up with that of the proteins already in the cycle (1, 15). When the degradation rate is high, the new proteins are likely to be degraded before the PPC can synchronize their phosphorylation levels; indeed, in the limit that the protein synthesis and decay rates go to infinity, the phosphorylation level $p(t)$ (defined as the fraction of phosphorylated monomers) becomes constant in time and equal to the phosphorylation level of freshly made KaiC proteins. This is not a purely stochastic effect; the white bifurcation line of Fig. 3 shows that even in a deterministic model the oscillations disappear when the synthesis and decay rates become too big (see *SI Text*).

C. *PPC-TTC*: A Protein Phosphorylation Cycle with a Transcription–Translation Cycle Is very Stable. To sustain a phosphorylation cycle, KaiC has to be made in an oscillatory fashion: Newly synthesized KaiC proteins should be injected into the phosphorylation cycle only when their phosphorylation state matches that of the PPC as a whole (1). This is the principal role of the transcription–translation cycle. Here, we present a mathematical model, the *PPC-TTC* model, for how such a cycle might interact with the PPC (Fig. 1*A*).

Although the TTC is much less well understood than the PPC, recently much progress has been made (37–39). In particular, several proteins important for transcriptional regulation of the *kaiBC* operon have now been identified. Our model is inspired by that of Taniguchi et al. (40) and contains the following key ingredients:

1. RpaA activates kaiBC expression. Deletion of *rpaA*, which encodes a putative response regulator, reduces *kaiBC* expression (40–42). Because neither promoters nor transcription or chromosome-compaction factors have been identified that interact with RpaA (42), we make the phenomenological assumption that RpaA directly activates *kaiBC* expression (40).

2. RpaA is activated by KaiC when KaiC is in the active state. RpaA is activated via phosphorylation by the histidine kinase SasA, whose activity is in turn stimulated by KaiC (38, 42); inactivation of SasA reduces *kaiBC* expression (40–42). Moreover, RpaA phosphorylation occurs 4–8 h before the peak of KaiC phosphorylation (42). This suggests that partially phosphorylated KaiC that is on the PPC's active branch activates RpaA through SasA (40). Because SasA phosphorylation, occurring on time scales of minutes (38), is much faster than KaiC phosphorylation, occurring on time scales of hours, we assume that the SasA dynamics can be integrated out.

3. RpaA is inactivated by KaiC when KaiC is in the inactive state. Inactivation of LabA (41) or CikA (40) increases *kaiBC* expression, with inactivation of both having a still stronger effect (40). SasA inactivation can compensate for both LabA (41) and CikA inactivation (40), but RpaA inactivation cannot compensate for LabA inactivation (41). Taken together, these results suggest that SasA, LabA, and CikA control *kaiBC* expression through different pathways, with at least the SasA and LabA pathways converging on RpaA (40). Because phosphorylation of KaiC is critical for negative feedback on *kaiBC* expression (10), LabA and CikA appear to act downstream of phosphorylated KaiC (40). Because the mechanisms by which LabA and/or CikA repress RpaA activation are unknown, we make the phenomenological assumption that inactive KaiC deactivates RpaA.
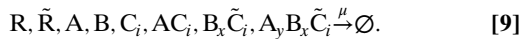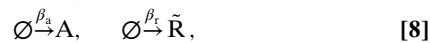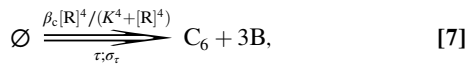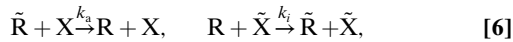
4. KaiC is injected into the system as fully phosphorylated hexamers. Imai et al. reported that newly synthesized KaiC is phosphorylated in vivo within 30 min (36), much faster than phosphoryla-

tion of KaiC hexamers in vitro, which takes about 6 h (12); we thus assume that newly synthesized KaiC is injected into the system as fully phosphorylated KaiC hexamers.

5. KaiA and RpaA are synthesized at constant rates. The mRNA levels of *kaiA* and *rpaA* exhibit much weaker oscillations than those of *kaiB* and *kaiC* (43). We therefore assume that *kaiA* and *rpaA* are expressed at constant rates.

6. The phosphorylation cycle in vivo is similar to that in vitro.
Fig. 1*A* shows a cartoon of this model, which is described by the reactions of formulas **1–5** for the PPC together with the following reactions for the TTC and the coupling between them:

$$\tilde{R} + X \xrightarrow{k_a} R + X, \qquad R + \tilde{X} \xrightarrow{k_i} \tilde{R} + \tilde{X}, \qquad \text{[6]}$$

$$\varnothing \underset{\tau;\sigma_\tau}{\overset{\beta_c[R]^4/(K^4+[R]^4)}{\rightleftharpoons}} C_6 + 3B, \qquad \text{[7]}$$

$$\varnothing \xrightarrow{\beta_a} A, \qquad \varnothing \xrightarrow{\beta_r} \tilde{R}, \qquad \text{[8]}$$

$$R, \tilde{R}, A, B, C_i, AC_i, B_x\tilde{C}_i, A_yB_x\tilde{C}_i \xrightarrow{\mu} \varnothing. \qquad \text{[9]}$$

Formula **6** models activation of inactive RpaA, $\tilde{R}$, by $X \in \{AC_2,...AC_5\}$ and inactivation of active RpaA, R, by $\tilde{X} \in \{A_yB_x\tilde{C}_5...A_yB_x\tilde{C}_2\}$ (see *SI Text*). Formula **7** models activation of *kaiBC* by RpaA, using a Hill function with coefficient 4. We assume a normally distributed delay, denoted by the double arrow, with mean $\tau = 5$ h and standard deviation $\sigma_\tau = 0.5$ h, between the activation of *kaiBC* transcription and the appearance of KaiB and KaiC protein. The length of the delay is dictated by the requirement that fresh KaiC be produced when its phosphorylation state matches that of the PPC. Formula **8** models constitutive expression of *kaiA* and *rpaA*. Formula **9** describes degradation of all species with the same rate constant μ; we ignore rhythmic KaiC degradation (36), which is not essential to produce a robust clock. In *SI Text* we show that, because of the stabilizing effect of the PPC, the clock's basic behavior is robust to variations in parameters such as the size of gene expression bursts, the width of the delay distribution, and the Hill coefficient of gene repression; there, we also discuss a more detailed model that includes cell growth and binomial partitioning upon cell division, which appreciably increases noise without changing qualitative trends. Although one can think of our model in loose terms as consisting of coupled transcriptional and posttranslational oscillators, it cannot be mathematically decomposed into two separate oscillatory systems, each with its own variables, nor can the strength of the coupling between the two cycles be independently tuned. It is thus formally quite different from textbook models of coupled oscillators (44).

Fig. 4 shows the robustness of this PPC-TTC model as a function of cell volume and protein degradation rate μ. As expected, $n_{1/2}$ decreases with decreasing cell volume. Its dependence on the degradation rate, however, is markedly different from that seen with constant KaiC synthesis (Fig. 3): A PPC sustained by a TTC becomes *more* robust with increasing decay rate. If we assume that proteins are lost only through dilution, then for a bacterial volume of 1 μm³ and a cell doubling time of 24 h (corresponding to a decay rate $\mu = 0.03$ h⁻¹), the correlation time is about 200 days, consistent with the value measured experimentally (5). If proteins are also degraded actively, increasing μ, this excellent behavior improves still further; even for $V = 0.5$ μm³, $n_{1/2} \approx 120$ for $\mu = 0.1$ h⁻¹. This is in stark contrast to the stability of a PPC without a TTC (Fig. 3).

D. TTC-only: A Protein Phosphorylation Cycle Dramatically Enhances the Robustness of a Transcription–Translation Cycle. Figs. 3 and 4
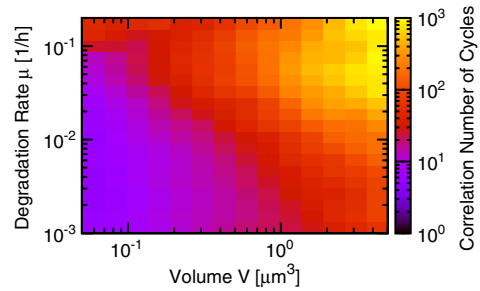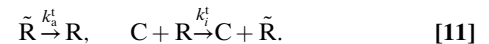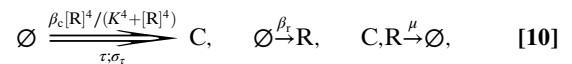

Fig. 4. *PPC-TTC* model (Fig. 1*A*). Correlation number of cycles, $n_{1/2}$, as a function of the volume *V* and degradation rate μ. The combination of a TTC and a PPC can generate stable circadian rhythms for a bacterial volume of $V \sim 1$ μm³, even when the degradation rate is high (compare Fig. 3). The protein synthesis rates are varied with the degradation rates such that the average protein concentrations equal those used in vitro (see Fig. 2). For time traces of $p(t)$ and $[C](t)$, see Fig. S1.

show that a TTC can greatly improve the robustness of a PPC. One might thus ask whether the PPC is needed at all, or whether an adequate clock can be built with only a TTC. To address this question, we modify the PPC-TTC model (formulas **6–9**) so that it consists only of a TTC, leading to the *TTC-only* model (Fig. 1*D*):

$$\varnothing \underset{\tau;\sigma_\tau}{\overset{\beta_c[R]^4/(K^4+[R]^4)}{\rightleftharpoons}} C, \qquad \varnothing \xrightarrow{\beta_r} R, \qquad C,R \xrightarrow{\mu} \varnothing, \qquad \text{[10]}$$

$$\tilde{R} \xrightarrow{k_a^t} R, \qquad C + R \xrightarrow{k_i^t} C + \tilde{R}. \qquad \text{[11]}$$

In the simulations, we adjust not only the synthesis rate β_c but also the delay τ so that both the average concentrations and the clock period remain constant when we vary μ (see *SI Text*).

Fig. 5 shows, for a bacterial volume of 1 μm³, the robustness of this *TTC-only* model as a function of the degradation rate, together with the results for the *PPC-TTC* model (Fig. 4) and the *PPC-in vivo* model (Fig. 3). The *TTC-only* model's behavior is the opposite of that of the *PPC-in vivo* model; the *TTC-only* model is most stable for *high* degradation rates, and its robustness falls dramatically when μ drops below 0.2 h⁻¹. The combined *PPC-TTC* model, however, is robust for all degradation rates—its correlation time interpolates between those of the *TTC-only* model for large μ and of the *PPC-in vivo* model for small μ. Importantly, the relative advantage of the combined model is greatest when $1/\mu$ is of order the oscillator period, and this is precisely the regime where physiological degradation and dilution rates for *S. elongatus* fall (36). Further, the combined oscillator does much
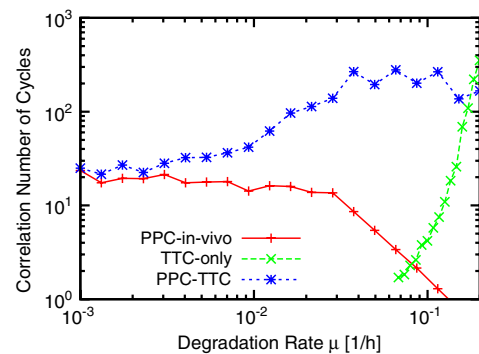

Fig. 5. Correlation number of cycles $n_{1/2}$ as a function of degradation rate μ for the *PPC-in vivo* (Fig. 1*C*), *TTC-only* (Fig. 1*D*), and *PPC-TTC* (Fig. 1*A*) models. $V = 1$ μm³ in all models; for the TTC, $n_{1/2}$ was computed for $[C](t)$ rather than for $p(t)$ (see *SI Text*). Clearly, a PPC in combination with a TTC generates robust rhythms over a wide range of degradation rates. The protein synthesis rates are varied with the degradation rates such that the average concentrations equal those used in the in vitro experiments (see Fig. 2).

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better than either oscillator alone; in contrast, when two conventional phase oscillators with comparable noise levels are coupled, one expects only about a factor of two gain in $n_{1/2}$ (44).

Dilution puts a lower bound on the degradation rate, which means that a stable oscillator cannot be based on a PPC only, especially when the growth rate of the bacterium is large. The degradation rate can, on the other hand, be increased by active degradation. For high degradation rates, i.e., $\mu > 0.2$ h^{-1}, an oscillator based only on a TTC can be sufficiently robust (Fig. 5). However, to balance these high decay rates, the protein synthesis rates have to be correspondingly large, which can be energetically costly (45). Combining a PPC with a TTC makes it possible to dramatically improve the robustness while keeping the synthesis rates the same. Although a phosphorylation cycle does not come entirely for free (46), this suggests that a PPC combined with a TTC gives the best performance-to-cost ratio.

E. The Principal Pacemaker. What drives the circadian clock, the PPC or the TTC (1)? As our analysis shows, the answer depends on the growth rate. When protein turnover is slower than the oscillation period, the clock is primarily driven by the PPC, and adding a TTC does not enhance its robustness (Fig. 5). Indeed, a TTC can generate large-amplitude oscillations in KaiC concentration only if proteins are synthesized and degraded on time scales faster than the oscillation period. At high growth rates, on the other hand, adding a PPC to a TTC does not improve stability, and the TTC is the principal driver. In this regime, the time scale for protein turnover is much faster than that for protein (de)phosphorylation; KaiC hexamers are thus typically degraded before they can complete a single phosphorylation cycle, rendering the PPC irrelevant.

To gain a deeper understanding of the transition between these two limiting cases—and in particular of the crossover regime where $1/\mu$ is of order 24 h and the gain in robustness from combining TTC and PPC is most dramatic—we introduce in *SI Text* section S2 minimal versions of the models discussed above together with two additional models designed to isolate specific modes of interaction between the two cycles (see also Fig. S2). The first of these, the *PMS-TTC* model, combines a TTC with a protein-modification sequence (PMS): the KaiC hexamers undergo a sequence of dephosphorylation steps on the PPC's inactive branch, but not a full phosphorylation cycle. The second additional model, the *UPPC-TTC* model, combines a TTC with an unsynchronized protein phosphorylation cycle (UPPC); here, each KaiC can undergo a full cycle of protein-modification steps as in the original PPC, but no KaiA is present, and synchronization through differential affinity thus cannot occur. These two models reveal two distinct mechanisms by which coupling a TTC with a PPC enhances robustness in the crossover regime (Fig. S3). The *PMS-TTC* model shows that the sequence of Poissonian protein-modification steps, an inherent property of the PPC, narrows the distribution of the delay between gene repression and gene expression, which enhances the robustness of the TTC. The *UPPC-TTC* model shows that the TTC is not only necessary to sustain a PPC at higher growth rates, as discussed above, but in fact enhances it, because the periodic synthesis of KaiC in a given phosphorylation state has a synchronizing effect on the phosphorylation cycles of the individual KaiC hexamers.

The picture that emerges from the analysis of these models is thus the following: At small μ, the PPC functions as it does in vitro, with protein synthesis and degradation playing only a minor role. As μ increases, the burden of synchronization is gradually passed from the differential affinity mechanism to periodic protein synthesis; we can still usefully think of the oscillation as driven by the PPC, but with the TTC now lending an essential helping hand. Finally, at still larger μ, $1/\mu$ becomes much larger than the oscillation period, and most hexamers do not survive long enough to complete a full phosphorylation cycle. At this point, the clock has become essentially a standard delayed negative-feedback oscillator, with the twist that, unless μ is very large, the PPC helps to narrow the distribution of delay times and thus to reduce noise. Consistent with recent experiments (16), our simulations suggest that *S. elongatus* usually finds itself in the second of these regimes, in which the TTC plays an important part in synchronizing the PPC.

Discussion

The evidence is accumulating that circadian rhythms are driven by both transcription–translation and protein-modification cycles not just in cyanobacteria, but even in higher organisms (1, 47). Our analysis suggests that both cycles are required to generate stable circadian rhythms in growing, dividing cells over a wide range of conditions: Although a PPC alone must inevitably fail at high and a TTC without active degradation at low growth rates, the combined clock is robust for all growth rates. Although our *PPC-TTC* model is simplified, it does capture the essence of a coupled TTC and PPC. That is, it is built around a protein that not only undergoes a protein-modification cycle, but also regulates its own synthesis in a manner that depends on its modification state. Moreover, its qualitative behavior can be explained by the generic arguments of the preceding section, which are based on a simple comparison of the clock period with the time scales of protein modification and protein degradation. Our results should thus apply to any system that exploits both a PPC and a TTC to generate circadian rhythms.

Our combined *PPC-TTC* model is consistent with a number of experimental observations. It not only matches the average oscillations of the phosphorylation level and the total KaiC concentration in wild-type cells (Fig. S1), but also reproduces the observation that even in the presence of an excess of KaiA, the total KaiC concentration still undergoes at least a damped oscillation with a circadian period (14, 16) (see Fig. S4). Yet, because quite a few elements of the TTC have not been characterized experimentally, our model of the TTC is necessarily rather simplified and phenomenological; not surprisingly, some observations thus cannot be reproduced. For example, our model predicts that the phase of the oscillation in KaiC abundance lags behind that of the phosphorylation level by a few hours, whereas experiments seem to show that these oscillations are more in phase (12, 14). This may be due to our simplifying assumption that KaiC is produced as fully phosphorylated hexamers. Although phosphorylation of fresh KaiC has been reported to occur within 30 min (36), fragmentary evidence suggests that hexamerization is slow (48); one would then expect to detect KaiC monomers in experiments several hours before our simulations report the presence of fully functional hexamers.

In *S. elongatus*, the period of the circadian rhythm is insensitive to changes in the growth rate (49, 50). Because protein synthesis and decay rates, on the other hand, tend to vary with the growth rate (51), the question arises whether the period of the oscillator as predicted by our model is robust to such variations. In *SI Text* we show that a tenfold increase in the synthesis and degradation rates of all proteins decreases the period only by 10–20%. The reason is that the clock period is mostly determined by the intrinsic period of the PPC. The latter does not depend on the absolute protein synthesis and decay rates, although it does depend on the ratio of the concentrations of the Kai proteins (26, 33, 34). Clearly, it would be of interest to investigate how this ratio varies with the growth rate.

Finally, how could our predictions be tested experimentally? The most important testable prediction of our analysis is that the PPC requires a TTC when the growth rate is high. Kondo and coworkers have demonstrated that a PPC can function in the absence of a TTC (12). These experiments were performed under constant dark conditions, which means that the growth rate was (vanishingly) small, protein synthesis was halted, and even

protein decay was probably negligible, because the KaiC level was rather constant (12). These experiments are consistent with our predictions, but the critical test would be to increase the growth rate while avoiding any circadian regulation of *kaiBC* expression. This means that the cyanobacteria must be grown under constant light conditions. Moreover, one would need to bring *kaiBC* expression under the control of a promoter that is constitutively active. However, most promoters of *S. elongatus* (37, 43), and even many heterologous promoters from *Escherichia coli* (52), are influenced by the circadian clock. Nevertheless, a number of promoters have been reported that exhibit arrhythmic activity (43), and these might be possible candidates. A still more challenging experiment would be to express the phosphorylation cycle in the bacterium *E. coli* (53). Our analysis predicts that in growth-

arrested cells, the phosphorylation cycle should be functional, whereas in normal growing *E. coli* cells, with cell doubling times of roughly 1 h, the oscillations should cease to exist.

Methods

The simulations were performed using the algorithm of ref. 35, and $n_{1/2}$ was computed from the decay of the correlation function of $p(t)$, except for the *TTC-only* model, for which we used $[C](t)$; our estimates for $n_{1/2}$ are typically accurate to within 15–20% (see SI Text).

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