,

Discrete gene replication events drive coupling between the cell cycle and circadian clocks

Joris Paijmans,[1] Mark Bosman,[1] Pieter Rein ten Wolde,[1] and David K. Lubensky[2]

[1]*FOM Institute AMOLF, Science Park 104, 1098 XG Amsterdam, The Netherlands*

[2]*Department of Physics, University of Michigan, Ann Arbor, MI 48109-1040*

Abstract

Many organisms possess both a cell cycle to control DNA replication and a circadian clock to anticipate changes between day and night. In some cases, these two rhythmic systems are known to be coupled by specific, cross-regulatory interactions. Here, we use mathematical modeling to show that, additionally, the cell cycle generically influences circadian clocks in a non-specific fashion: The regular, discrete jumps in gene-copy number arising from DNA replication during the cell cycle cause a periodic driving of the circadian clock, which can dramatically alter its behavior and impair its function. A clock built on negative transcriptional feedback either phase locks to the cell cycle, so that the clock period tracks the cell division time, or exhibits erratic behavior. We argue that the cyanobacterium *Synechococcus elongatus* has evolved two features that protect its clock from such disturbances, both of which are needed to fully insulate it from the cell cycle and give it its observed robustness: a phosphorylation-based protein modification oscillator, together with its accompanying push-pull read-out circuit that responds primarily to the ratios of the different phosphoforms, makes the clock less susceptible to perturbations in protein synthesis; and the presence of multiple, asynchronously replicating copies of the same chromosome diminishes the effect of replicating any single copy of a gene.

Significance Statement: Huygens famously showed that two mechanically connected clocks tend to tick in synchrony. We uncovered a generic mechanism that can similarly phase lock two rhythmic systems present in many living cells: the cell cycle and the circadian clock. DNA replication during the cell cycle causes protein synthesis rates to show sharp, periodic jumps that can entrain the clock. To faithfully keep time in the face of these disturbances, circadian clocks must incorporate specific insulating mechanisms. We argue that, in cyanobacteria, the presence of multiple, identical chromosome copies and the clock's core protein-modification oscillator together play this role. Our results shed new light on the complex factors that constrain the design of biological clocks.

I. INTRODUCTION

Circadian clocks—autonomous oscillators with a roughly 24 hour period that can be entrained to daily cycles of light and dark —are thought to confer important advantages on living cells by allowing them to anticipate diurnal environmental changes. Recent decades have seen considerable progress in elucidating both the architecture and the function of these biological timekeepers. Circadian clocks, however, are not the only oscillatory systems present in living cells. Most notably, cell growth and division are governed by a cell cycle, which can in many contexts be viewed as an autonomous oscillator. Much recent attention has been directed towards the connections between these two rhythmic systems, which are relevant for processes ranging from plants' response to shade [1] to cancer susceptibility [2, 3]. In particular, it is now clear that circadian clocks can exert specific regulatory influences on the cell cycle, and a number of experimental and modeling studies have sought to tease out the implications of this regulation [4–11]. Here, we argue that, in addition to direct, specific regulation of one oscillator by the other, there must also be more generic connections between the circadian clock and the cell cycle [2, 10–12]. In particular, we focus on the consequences of the discrete gene replication events that accompany DNA replication. We show that, as a result of the regular jumps in gene copy number caused by these events, the cell cycle must, very generally, contribute a periodic forcing to the circadian clock. This forcing can markedly change clock behavior and degrade clock function. We propose that cyanobacterial clocks have evolved specific features that can mitigate this effect. More broadly, this generically strong coupling to the cell cycle implies important constraints on the design of biological timekeepers if they are to remain accurate in dividing cells.

It is widely accepted that protein levels depend on a cell's gene dosage. Typically, a doubling of the number of chromosomal copies of a gene should lead to an approximate doubling of its mRNA synthesis rate and thus to a corresponding increase in its protein levels. Most often, however, such effects are considered in the context of a change in the number of autosomal gene copies that persists throughout an organism's lifetime [13], as, e.g., in the haploinsufficiency of certain genes [14]. It is less often acknowledged that the number of copies of all genes varies over each cell cycle, despite evidence that these variations have measurable consequences [15–18]. Because of the well-known phenomenon of phase locking of oscillators [19], regular, periodic changes in gene dose are likely to be especially relevant

to cellular oscillators that depend on gene expression. A circadian clock that became slaved to the cell cycle, for example, would lose its identity as an autonomous timekeeper, and thus much of its ability to perform its biological function. Here, we show that oscillators built on negative transcriptional feedback—a common motif in both prokaryotic and eukaryotic clocks—are indeed very strongly affected by driving from periodic gene replication events. This immediately raises the question of how real biological clocks are able to function in growing, dividing cells. To address this, we study the circadian clock of the cyanobacterium *Synechococcus elongatus*, which is known to exhibit stable rhythms over a wide range of growth rates [20, 21], but whose clock appears not to regulate DNA replication [4], suggesting exactly the sort of unidirectional forcing of the clock by the cell cycle that might have been expected to impair clock function.

The *S. elongatus* clock combines a negative transcriptional feedback oscillator (the transcription-translation cycle, or TTC) with a core phosphorylation-based post-translational oscillator (the protein phosphorylation cycle, or PPC). Remarkably, the PPC can be reconstituted *in vitro* with purified proteins [22], allowing detailed study of the mechanisms behind its oscillation. A number of studies have begun to converge on the view that the PPC works by synchronizing the intrinsic phosphorylation cycles of individual KaiC hexamers, primarily through phosphorylation-dependent sequestration of KaiA by KaiC [23–29]. Although many details of the TTC remain murkier, it seems clear that the protein RpaA plays a central role, regulating the expression of clock components in a manner that depends on the KaiC phosphorylation state [30–33]. Depending on light and nutrient levels, *S. elongatus* can have doubling times ranging from 6 to 72 h [21]; the cell cycle period is thus of the same order as the clock period of roughly 24 h, opening the way for interactions between the two. Indeed, the circadian clock is known to gate mitosis, prohibiting cell division during certain clock phases [4, 7, 8], although in constant light this gating leaves both DNA replication and cell growth essentially unchanged [4]. Conversely, Mori and Johnson argued that cell growth and division don't affect the *S. elongatus* circadian clock [20]. We use mathematical modeling to study the unidirectional forcing of the clock by the cell cycle. We identify specific features of the *S. elongatus* clock that tend to insulate it from entrainment by regular gene replication events. Nonetheless, we argue that, under certain conditions, it should be possible to observe signatures of periodic forcing of the clock by the cell cycle. We further suggest how some of the clock's protective mechanisms might be weakened experimentally,

leading to much stronger signatures of its coupling to the cell cycle.

Below, we first model the effects of cell growth and division on a constitutively expressed protein. We show that gene replication, not cell division, is the essential cell-cycle event that influences protein concentrations and that, as long as the constitutively expressed protein is not subject to rapid, active degradation, its concentration varies little over the cell cycle. In contrast, gene replication can dramatically affect the behavior of a negative transcriptional feedback oscillator (NTFO): the NTFO locks to the cell cycle over a range of cell-division times of many hours and shows erratic behavior outside this regime[12]. We next ask how the real cyanobacterial clock can be so apparently undisturbed by the cell cycle. We find that incorporating both a PPC and a TTC into the clock significantly weakens coupling to the cell cycle, especially when the clock is read out by a push-pull network that is more sensitive to ratio of concentrations of different phosphorylation states than to their absolute values. The presence of multiple chromosome copies has a still more striking effect: If the cell has 4 copies after division (rather than only 1), as can often be the case in *S. elongatus*, and if these are replicated one after the other [34], then the dose of the clock genes changes much more gradually, and cell cycle effects are almost completely lost. Thus, *S. elongatus* may have evolved to carry multiple, identical chromosome copies in part to insulate its circadian clock from its DNA replication cycles.

II. MODELS AND RESULTS

A. The cell cycle's effect on a constitutively expressed gene is weak

Before turning to the more complex case of a circadian clock, we first investigate how the concentration of a single, constitutively expressed protein varies over a cell cycle. To this end, we add regular, rhythmic DNA replication and mitosis to a simple model of protein production and dilution.

The key quantities in our description are the number of copies $g(t)$ of the gene of interest and the cell volume $V(t)$. These vary periodically in time as sketched in Fig. 1A–B, with a period given by the cell division time T_{d}. We assume for now that there is only one gene copy present immediately after cell division. This copy is replicated at some time before the next division, at which point $g(t)$ jumps from 1 to 2. When the cell divides, the chromosomes are

split between the daughter cells, and $g(t)$ returns to 1. The cell volume grows exponentially: $V(t) = V_0 \exp(\mu_{\rm d} t)$, with $\mu_{\rm d} = \log(2)/T_{\rm d}$. When t reaches $T_{\rm d}$, division occurs, and $V(t)$ drops back from $2V_0$ to V_0.

The variables $g(t)$ and $V(t)$ define the gene density $G(t) \equiv g(t)/V(t)$. As long as noise and spatial variations are neglected, the behavior of a biochemical network depends only on protein *concentrations*, not separately on protein numbers and cell volume. As a result, the system responds to the protein synthesis rate per unit volume, proportional to $G(t)$, but not to $g(t)$ and $V(t)$ individually (Eq. 1, below). Fig. 1C shows that $G(t)$ has only a single discontinuity during the cell cycle, corresponding to the doubling of $g(t)$ when the gene is copied; at cell division, both $g(t)$ and $V(t)$ are halved, so their ratio is unchanged. Importantly, then, the meanfield, deterministic dynamics of a biochemical network is sensitive to the timing of DNA replication but not of cell division. This dynamics is likewise unaffected by any gating of cell division by the circadian clock, provided, as is the case in *S. elongatus* [4, 8], that this gating does not affect DNA replication or cell growth. Similarly, regardless of when during the division cycle the gene is copied, the time dependence of $G(t)$ is always the same: It doubles, decays exponentially for a time $T_{\rm d}$, then doubles again, etc. The exact moment of gene replication affects only the average value of $G(t)$, which can be absorbed, for modeling purposes, into the parameter β (Eq. 1, below). For simplicity, we thus always assume that the gene is replicated exactly at $t = T_{\rm d}/2$.

Given the gene density $G(t)$, the concentration $C(t)$ of a constitutively expressed protein evolves as:

$$\frac{dC(t)}{dt} = \beta G(t) - \mu_{\rm d} C(t). \tag{1}$$

Here, proteins are expressed at a rate β per gene copy and diluted by cell growth at a rate $\mu_{\rm d} = \log(2)/T$. We thus assume that, as is true for many bacterial proteins, the protein is not subject to active degradation [36]. Fig. 1D shows how $C(t)$ varies over the cell cycle. Remarkably, even though the protein production rate doubles each time the gene is replicated, the protein concentration varies by no more than a few percent: The discrete jumps in protein production are smoothed out by the slow protein dilution. Thus, a protein that is constitutively expressed and not actively degraded is little affected by the cell cycle.

B. The cell cycle strongly perturbs both the period and the amplitude of a negative transcriptional feedback oscillator

Although the concentration of a protein that is constitutively expressed does not vary much over the cell cycle, oscillators are known to be far more sensitive to periodic driving than non-oscillatory systems [19]. We thus next consider a simple model for a clock built on delayed, negative transcriptional feedback (Fig. 1E). The model consists of a single variable, $C(t)$, describing the concentration of proteins that inhibit their own production:

$$\frac{dC(t)}{dt} = \beta\,\tilde{G}(t)\,\frac{K_{\rm c}^n}{K_{\rm c}^n + C(t-\Delta)^n} - \mu_{\rm tot}C(t). \tag{2}$$

We impose a fixed delay Δ between the initiation of transcription and the appearance of functional proteins. Therefore, protein production at time t is proportional to the gene copy number $g(t-\Delta)$ at time $t-\Delta$. These proteins 'arrive' in the cell volume $V(t)$ at time t.The protein synthesis rate per unit volume at time t is thus proportional to the *protein production density* $\tilde{G}(t) \equiv g(t-\Delta)/V(t)$. $\tilde{G}(t)$ is a generalization of the gene density $G(t)$ of the preceding section to the case with a delay Δ and parametrizes the periodic forcing of the NTFO by gene replication. Proteins disappear with a total rate $\mu_{\rm tot} = \mu_{\rm d} + \mu_{\rm act}$, where as before $\mu_{\rm d}$ describes dilution due to cell growth, and $\mu_{\rm act}$ describes possible active degradation. Including both terms allows us to vary the doubling time $T_{\rm d}$ while holding $\mu_{\rm tot}$ constant and hence, in our simulations, to distinguish the trivial influence of the cell cycle on the clock through the dilution rate $\mu_{\rm d}$ from other effects.

We next define the peak-to-peak time $T_{\rm PtP}$ as the time between successive peaks in $C(t)$ (see Fig. 2 and *Supporting Information* [SI]); $T_{\rm PtP}$ reduces to the period of the circadian clock when oscillations are regular but remains defined when the cell cycle induces more erratic behavior. In Fig. 2A we plot the average peak-to-peak time $\langle T_{\rm PtP}\rangle$ for a range of division times $T_{\rm d}$ at fixed $\mu_{\rm tot}$.

As expected from the general theory of driven oscillators [19], the curve shows two striking features: First, around division times which are fractions or multiples of the clock's intrinsic period of 24 h, the cell cycle determines the period of the clock. Especially around $T_{\rm d} = 24$ and 48 h, the average peak to peak time is directly proportional to $T_{\rm d}$. At $T_{\rm d} = 24$ h (1:1 locking), $\langle T_{\rm PtP}\rangle = T_{\rm d}$, and the amplitude of each clock oscillation cycle is the same (Fig. 2B). At $T_{\rm d} = 48$ h (2:1 locking), however, $\langle T_{\rm PtP}\rangle = T_{\rm d}/2$, and two full clock cycles are required to

make up a single division time. Because these two cycles occur at different gene densities, successive peaks in the trace of $C(t)$ have alternately large and small amplitudes.

Second, the standard deviation of T_{PtP} becomes very large just outside the locking regions. Fig. 2C shows that this variability in the phase of $C(t)$ is accompanied by substantial fluctuations in the amplitude for $T_{\mathrm{d}} = 27$ h. Because the difference between T_{d} and the intrinsic clock period is just too large to allow stable locking, the clock constantly tries to lock to the cell cycle, but slips from time to time. As a result, the cell cycle dramatically disrupts the clock. In the *SI* we show that both of these effects survive the introduction of intrinsic noise in chemical reactions and of stochasticity in the timing of DNA replication (Figs. S1–2; see also Fig. S6). Fig. 3 qualitatively explains how locking arises in the NTFO.

C. A phosphorylation cycle makes the clock more robust against a time-varying gene density

To study how a more realistic clock can become resilient to variability in the gene density, we turn to the *S. elongatus* circadian clock, and more specifically to the model of Zwicker *et al.* [25, 35] (Fig. 1F). This model provides a detailed description of the clock, including the synchronization of the phosphorylation state of different KaiC hexamers via KaiA sequestration and the coupling of the PPC oscillator to the TTC via RpaA. It represents KaiC as a hexamer but does not explicitly take into account that each KaiC monomer has two distinct phosphorylation sites [26, 37]. In the *SI Text* we show that a model based on that of Rust *et al.* [26], which describes KaiC at the level of monomers with two phosphorylation sites, gives similar results. We thus expect that still more elaborate models of the PPC, which include hexameric KaiC with two phosphorylation sites per monomer [29], will lead to similar results. To include gene replication, we modify the model of [35] so that the delayed negative feedback on KaiC production is modulated by a regularly oscillating protein production density $\tilde{G}(t)$ (see *SI Text*). We follow both the total KaiC concentration $C_{\mathrm{tot}}(t)$ and the KaiC phosphorylation fraction $p(t) = \sum_{n=1}^{6} nC_n(t)/(6\,C_{\mathrm{tot}}(t))$, where C_n is the concentration of n-fold phosphorylated KaiC hexamers.

Fig. 4A shows that a model with a PPC coupled to a TTC has a smaller locking window than an NTFO and lacks the large deviations in T_{PtP} just outside the locking region. The *S. elongatus* clock is hence more robust to gene replication than one based only on negative

transcriptional feedback.

D. Clock readout through an RpaA-based push-pull network filters out cell-cycle-dependent variations in protein concentrations

Although the variance of T_{PtP} outside of the locking region is relatively small for the combined TTC-PPC model, Fig. 4B shows that $C_{\mathrm{tot}}(t)$ exhibits strong amplitude fluctuations, mirroring those observed for the NTFO (Fig. 2). The phosphorylation fraction $p(t)$, in contrast, is far more resilient, suggesting that the clock encodes temporal information more reliably in $p(t)$ than in $C_{\mathrm{tot}}(t)$. Intriguingly, the RpaA-centered push-pull network that transmits this timing signal to downstream genes [30–33, 38] in fact responds primarily to $p(t)$: Because the rates of RpaA phosphorylation and dephosphorylation are controlled by different KaiC phosphoforms, variations in C_{tot} at fixed p change both rates together, leaving the fraction of phosphorylated RpaA largely unaffected. In contrast, changes in p shift the balance between the two opposing reactions and so modify the RpaA phosphorylation fraction (Fig. S4 and *SI text*). Thus, not only is the basic PPC-based timekeeping mechanism insulated from variations in protein synthesis, but the readout mechanism selectively follows this more robust signal.

E. Multiple chromosome copies weaken the cell cycle's influence on the clock

While the PPC reduces gene replication's effect on the clock, it does not eliminate it entirely. What other mechanisms might explain the observed resistance of the *S. elongatus* clock to cell-cycle locking? It is known that *S. elongatus* has multiple, identical copies of its chromosome [34, 39–41]. These are not duplicated simultaneously, but rather one at a time, so that DNA replication occurs at a roughly constant rate throughout the cell cycle; furthermore, the timing of chromosome duplication appears to be independent of the phase of the clock [4, 34, 39, 41, 42]. Motivated by this observation, we consider a situation in which a cell starts with N chromosomes after division and let $g(t)$ rise to $2N$ in N evenly spaced steps (Fig. 5A). Fig. 5B shows the corresponding gene density $G(t)$. Clearly, for higher N, the gene-copy number $g(t)$ increases more gradually, and hence the discrete jumps in $G(t)$ are considerably smaller. The effect on the clock is dramatic: The locking regions

almost disappear and the standard deviation in T_{PtP} becomes negligible (Fig. 5C). Multiple chromosomes similarly make the NTFO much less susceptible to gene replication, but in the absence of the PPC cell-cycle effects are not blocked so completely (Fig. S5). Fig. S6 summarizes the combined effects of chromosome number and variability in gene replication time on our clock models. Importantly, at small N gene replication always significantly affects the clock, through either phase locking or high variability in T_{PtP}.

III. DISCUSSION

Given the pleiotropic roles of both the cell cycle and the circadian clock, it is natural to ask whether they also influence each other. Our central observation is that such influence need not involve specific interactions between the core genes or proteins of the two systems [2, 10, 11]; rather, the simple fact that the number of cellular copies of a given gene necessarily experiences discrete jumps during DNA replication (Fig. 1) implies that clocks must in general feel a periodic driving from the cell cycle [12]. Whereas some genetic circuits can simply average over this time-varying input, oscillators—including biological clocks—are known to be especially sensitive to rhythmic forcing. Indeed, an NTFO either locks to the cell cycle or shows erratic oscillations for a range of doubling times T_{d} (Fig. 2), losing its ability to function as a clock in either case.

In light of this strong and detrimental coupling between the cell cycle and a simple transcriptional clock, it is all the more striking that the *S. elongatus* clock is so stable. Our analysis highlights two features of the cyanobacterial clock that are predicted to allow the necessary decoupling from the cell cycle. First, a time-varying gene dosage influences a clock with an autonomous post-translational oscillator less than it does a purely transcriptional clock; even *within* the combined TTC-PPC, the oscillations of the KaiC phosphorylation fraction $p(t)$ are less affected by periodic gene replication than are those of the total KaiC concentration $C_{\mathrm{tot}}(t)$ (Fig. 4, S2C). Strikingly, the RpaA-based push-pull network that communicates the clock state to the rest of the cell responds to p while ignoring the more strongly fluctuating C_{tot} (somewhat in the spirit of mechanisms that improve the robustness of bacterial chemotaxis to gene expression noise [43]). This filtering function of the push-pull architecture could help explain why the *S. elongatus* clock has a relatively complex output mechanism requiring both CikA and SasA rather than a simpler linear design [44].

The second feature of the *S. elongatus* clock that we predict mitigates perturbations from the cell cycle is the presence of multiple, identical, asynchronously replicating chromosome copies [34, 39, 41, 42]. This reduces the importance of each individual gene replication event: Rather than seeing a single doubling of the number of gene copies each cell cycle, a cell with many chromosomes instead sees a number of smaller jumps that it can more easily ignore (Fig. 5). This adaptation may thus have evolved in part to protect the *S. elongatus* clock from cell cycle effects.

Whereas we have argued that the cell cycle generically affects any transcriptional clock, no comparably general mechanisms exist in the other direction. Moreover, though in many eukaryotic systems the clock is known to regulate key cell-cycle genes [2–9, 45], no similar, specific connections have yet been characterized in *S. elongatus*. In particular, clock-dependent cell-cycle gating [4], because it acts on cell division but not on growth or DNA replication, does not allow the clock to block the discrete gene replication events that underlie the driving. Nonetheless, since the majority of *S. elongatus* genes shows some degree of clock-dependent expression [46], it is possible that the cyanobacterium's clock does regulate its cell cycle in some as yet undiscovered way. Any such coupling would however have to be weak enough to be consistent with the observation that the rhythm of DNA replication does not depend on clock phase [4, 34, 39, 41, 42]. Because phase locking between two oscillators has strong similarities to the locking of a single oscillator to periodic driving [19], most of our qualitative conclusions would remain unchanged in this case.

To isolate the behavior of the core, autonomous circadian oscillator, studies in the lab are typically performed at constant light levels. In keeping with this tradition, we have limited ourselves here to models of free-running clocks, without any diurnal environmental variation. In nature, however, the circadian clock is exposed to many additional entrainment signals, most notably the 24 h light-dark cycle. In fact, the environmental and cell cycle entrainment signals are intricately intertwined, because DNA replication and the synthesis of most proteins, including clock components, come to a standstill in the dark in a clock-independent fashion [42, 47]. We leave the effects of this complex interplay for future work.

Although we have focused on interactions between the cell cycle and the clock in *S. elongatus*, the basic idea that periodic gene replications must influence biological oscillators is more general and should apply to a wide range of prokaryotic and eukaryotic species. Indeed, cell-cycle-dependent changes in gene copy number have clearly observable effects on

gene expression in eukaryotic cells [16], and recent experiments in cultured metazoan cells strongly suggest that the cell cycle exerts a considerable influence on the circadian clock, generally leading to phase locking of the two oscillators [10, 11]. Other generic forms of driving from the cell cycle may also play a role here: for example, in contrast to prokaryotes, eukaryotes typically shut down transcription around mitosis, thereby introducing another source of periodic, cell-cycle dependent variation in protein synthesis [2, 10, 11]. Our analysis thus highlights an important constraint on the design of circadian clocks in organisms from bacteria to humans.

Further, there is no reason for the effects of regular, discrete gene replications to be limited to circadian clocks; they should be observable in any cellular oscillator that depends on transcription and has a period on the same order as that of the cell cycle. Thus, our results may be relevant to phenomena like coupling between the cell cycle and the segmentation clock in vertebrate development [48]. Similarly, in the *SI* (Figs. S7–S8) we show that two well-known synthetic circuits [49, 50] can also lock to the cell cycle, and that the strength of locking depends sensitively on the oscillator architecture.

Since we have argued that *S. elongatus* possesses particular adaptations that decouple its circadian clock from the cell cycle, the most obvious experimental test of our ideas would be to observe the consequences of blocking or removing these features. Several strains already exist that might allow just such experiments. Mutants of *S. elongatus* are known with significantly fewer chromosomes per cell than the wildtype [51]; moreover, in some other *Synechococcus* strains, cells are always monoploid [40]. We find that in cells where the number of chromosomes goes from 1 to 2 over the course of a single division cycle, it should be possible to observe clear signatures of driving by the cell cycle in plots of KaiC's abundance—but not its phosphorylation level—as a function of time (Fig. 4). We predict that this effect will be further strengthened if the PPC is removed entirely. It is well-established that this can be accomplished by hyper-phosphorylating KaiC [52, 53]. In all cases, one could study forcing by the cell cycle at a variety of different doubling times. We suggest, however, that a doubling time near 48 hours offers a particularly unambiguous signature of the cell cycle's influence: The KaiC abundance as a function of time should then rise and fall every 24 hours, with successive peaks strictly alternating between higher and lower levels (Fig. 4C).

Acknowledgments

We thank Jeroen van Zon for a critical reading of the manuscript. This work was supported in part by FOM, which is financially supported by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (JP, MB and PRtW), and by NSF Grant DMR-1056456 (DKL).

---

[1] Salter MG, Franklin KA, Whitelam GC (2003) Gating of the rapid shade-avoidance response by the circadian clock in plants. *Nature* 426:680–3.

[2] Johnson C (2010) Circadian clocks and cell division: What's the pacemaker? *Cell Cycle* 9:3864–3873.

[3] Sotak M, Sumova A, Pacha J (2014) Cross-talk between the circadian clock and the cell cycle in cancer. *Ann Med* 46:221–32.

[4] Mori T, Binder B, Johnson CH (1996) Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proc Natl Acad Sci USA* 93:10183–8.

[5] Matsuo T, et al. (2003) Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 302:255–9.

[6] Nagoshi E, et al. (2004) Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* 119:693–705.

[7] Dong G, et al. (2010) Elevated ATPase activity of KaiC applies a circadian checkpoint on cell division in Synechococcus elongatus. *Cell* 140:529–39.

[8] Yang Q, Pando BF, Dong G, Golden SS, van Oudenaarden A (2010) Circadian gating of the cell cycle revealed in single cyanobacterial cells. *Science* 327:1522–1526.

[9] Gerard C, Goldbeter A (2012) Entrainment of the mammalian cell cycle by the circadian clock: modeling two coupled cellular rhythms. *PLoS Comp Biol* 8:e1002516.

[10] Feillet C, et al. (2014) Phase locking and multiple oscillating attractors for the coupled mammalian clock and cell cycle. *Proc Natl Acad Sci USA* 111:9828–33.

[11] Bieler J, et al. (2014) Robust synchronization of coupled circadian and cell cycle oscillators in single mammalian cells. *Mol Syst Biol* 10:739.

[12] Bosman M (2012) Master's thesis (Universiteit van Amsterdam).

[13] Volfson D, et al. (2006) Origins of extrinsic variability in eukaryotic gene expression. *Nature* 439:861–4.

[14] Irish VF, Gelbart WM (1987) The decapentaplegic gene is required for dorsal-ventral patterning of the Drosophila embryo. *Genes Dev* 1:868–79.

[15] Trcek T, Larson DR, Moldon A, Query CC, Singer RH (2011) Single-molecule mRNA decay measurements reveal promoter- regulated mRNA stability in yeast. *Cell* 147:1484–97.

[16] Zopf CJ, Quinn K, Zeidman J, Maheshri N (2013) Cell-cycle dependence of transcription dominates noise in gene expression. *PLoS Comp Biol* 9:e1003161.

[17] Narula J, et al. (2015) Chromosomal Arrangement of Phosphorelay Genes Couples Sporulation and DNA Replication. *Cell* 162:328–337.

[18] Hensel Z, Marquez-Lago TT (2015) Cell-cycle-synchronized, oscillatory expression of a negatively autoregulated gene in E. coli. *arxiv.1506.08596v1.*

[19] Pikovsky A, Rosenblum M, Kurths J (2003) *Synchronisation: A universal concept in nonlinear sciences* (Cambridge University Press, Cambridge).

[20] Mori T, Johnson CH (2001) Independence of circadian timing from cell division in cyanobacteria. *J Bact* 183:2439–2444.

[21] Teng SW, Mukherji S, Moffitt JR, de Buyl S, O'Shea EK (2013) Robust circadian oscillations in growing cyanobacteria require transcriptional feedback. *Science* 340:737–40.

[22] Nakajima M, et al. (2005) Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science* 308:414–5.

[23] Kageyama H, et al. (2006) Cyanobacterial circadian pacemaker: Kai protein complex dynamics in the kaic phosphorylation cycle in vitro. *Mol Cell* 23:161–171.

[24] Clodong S, et al. (2007) Functioning and robustness of a bacterial circadian clock. *Mol Syst Biol* 3:90.

[25] van Zon JS, Lubensky DK, Altena PRH, ten Wolde PR (2007) An allosteric model of circadian KaiC phosphorylation. *Proc Natl Acad Sci USA* 104:7420–5.

[26] Rust MJ, Markson JS, Lane WS, Fisher DS, O'Shea EK (2007) Ordered phosphorylation governs oscillation of a three-protein circadian clock. *Science* 318:809–12.

[27] Brettschneider C, et al. (2010) A sequestration feedback determines dynamics and temperature entrainment of the KaiABC circadian clock. *Mol Syst Biol* 6:1–10.

[28] Qin X, et al. (2010) Intermolecular associations determine the dynamics of the circadian KaiABC oscillator. *Proc Natl Acad Sci USA* 107:14805–14810.

[29] Lin J, Chew J, Chockanathan U, Rust MJ (2014) Mixtures of opposing phosphorylations within hexamers precisely time feedback in the cyanobacterial circadian clock. *Proc Natl Acad Sci USA* 111:E3937–E3945.

[30] Takai N, et al. (2006) A KaiC-associating SasA-RpaA two-component regulatory system as a major circadian timing mediator in cyanobacteria. *Proc Natl Acad Sci USA* 103:12109–14.

[31] Taniguchi Y, Takai N, Katayama M, Kondo T, Oyama T (2010) Three major output pathways from the KaiABC-based oscillator cooperate to generate robust circadian kaiBC expression in cyanobacteria. *Proc Natl Acad Sci USA* 107:3263–8.

[32] Gutu A, O'Shea EK (2013) Two antagonistic clock-regulated histidine kinases time the activation of circadian gene expression. *Mol Cell* 50:288–294.

[33] Markson JS, Piechura JR, Puszynska AM, O'Shea EK (2013) Circadian control of global gene expression by the cyanobacterial master regulator RpaA. *Cell* 155:1396–408.

[34] Jain IHI, Vijayan V, O'Shea E (2012) Spatial ordering of chromosomes enhances the fidelity of chromosome partitioning in cyanobacteria. *Proc Natl Acad Sci USA* 109:13638–13643.

[35] Zwicker D, Lubensky DK, ten Wolde PR (2010) Robust circadian clocks from coupled protein-modification and transcription translation cycles. *Proc Natl Acad Sci USA* 107:22540–22545.

[36] Lengeler JW, Drews G, Schlegel HG (1999) *Biology of the Prokaryotes, p. 481* (Georg Thieme Verlag), p 481.

[37] Nishiwaki T, et al. (2007) A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in cyanobacteria. *EMBO J* 26:4029–4037.

[38] Goldbeter A, Koshland DE (1981) An amplified sensitivity arising from covalent modification in biological systems. *Proc. Natl. Acad. Sci.* 78:6840–6844.

[39] Binder BJ, Chisholm SW (1990) Relationship between DNA cycle and growth rate in Synechococcus sp. strain PCC 6301. *J Bact* 172:2313–9.

[40] Griese M, Lange C, Soppa J (2011) Ploidy in cyanobacteria. *FEMS Micr Lett* 323:124–31.

[41] Chen AH, Afonso B, Silver Pa, Savage DF (2012) Spatial and temporal organization of chromosome duplication and segregation in the cyanobacterium Synechococcus elongatus PCC 7942. *PLOS ONE* 7:e47837.

[42] Watanabe S, et al. (2012) Light-dependent and asynchronous replication of cyanobacterial multi-copy chromosomes. *Mol Micr* 83:856–865.

[43] Kollmann M, Lovdok L, Bartholome K, Timmer J, Sourjik V (2005) Design principles of a bacterial signalling network. *Nature* 438:504–507.

[44] Shultzaberger RK, Boyd JS, Katsuki T, Golden SS, Greenspan RJ (2014) Single mutations in sasA enable a simpler Delta-cikA gene network architecture with equivalent circadian properties. *Proc Natl Acad Sci USA* 111:E5069–75.

[45] Masri S, Cervantes M, Sassone-Corsi P (2013) The circadian clock and cell cycle: interconnected biological circuits. *Curr Opin Cell Biol* 25:730–4.

[46] Ito H, et al. (2009) Cyanobacterial daily life with Kai-based circadian and diurnal genome-wide transcriptional control in Synechococcus elongatus. *Proc Natl Acad Sci USA* 106:14168–14173.

[47] Tomita J, Nakajima M, Kondo T, Iwasaki H (2005) No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science* 307:251–4.

[48] Delaune EA, Francois P, Shih NP, Amacher SL (2012) Single-cell-resolution imaging of the impact of Notch signaling and mitosis on segmentation clock dynamics. *Dev Cell* 23:995–1005.

[49] Elowitz MB, Leibler S (2000) A synthetic oscillatory network of transcriptional regulators. *Nature* 403:335–338.

[50] Stricker J, et al. (2008) A fast, robust and tunable synthetic gene oscillator. *Nature* 456:516–520.

[51] Bird AJ, Turner-Cavet JS, Lakey JH, Robinson NJ (1998) A carboxyl-terminal Cys2/His2-type zinc-finger motif in DNA primase influences DNA content in Synechococcus PCC 7942. *J Biol Chem* 273:21246–52.

[52] Kitayama Y, Nishiwaki T, Terauchi K, Kondo T (2008) Dual KaiC-based oscillations constitute the circadian system of cyanobacteria. *Genes Dev* 22:1513–1521.

[53] Qin X, Byrne M, Xu Y, Mori T, Johnson CH (2010) Coupling of a core post-translational pacemaker to a slave transcription/translation feedback loop in a circadian system. *PLoS Biol* 8:e1000394.

[54] Bogan JA, et al. (2001) P1 and nr1 plasmid replication during the cell cycle of escherichia coli. *Plasmid* 45:200 – 208.

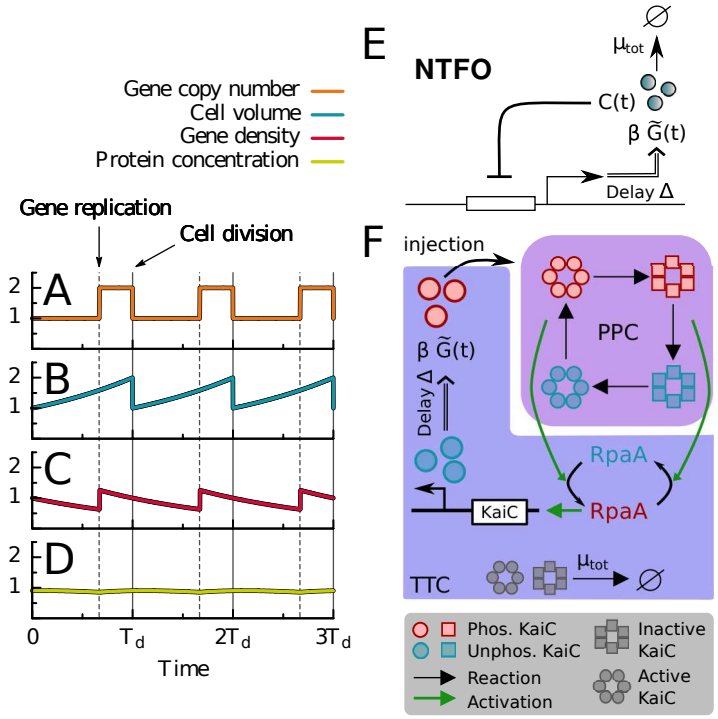

FIG. 1. DNA replication but not cell division affects average expression levels; for a protein that is constitutively expressed and decays by dilution only, the effect is small. Schematic time courses of the gene copy number $g(t)$ (A), the cell volume $V(t)$ (B), the gene density, $G(t) = g(t)/V(t)$ (C), and the concentration $C(t)$ of a constitutively expressed protein that decays only by dilution (D). Time in units of the cell division time $T_{\rm d}$; vertical axes, arbitrary units. The gene density (C) has a discontinuity when the gene is replicated (vertical dotted lines) but not at cell division (vertical solid lines), when both $g(t)$ and $V(t)$ are halved. Even though the protein synthesis rate doubles when the gene is replicated, the maximum deviation of $C(t)$ from its time average is less than 4% (D). (E) The NTFO model: A protein with concentration $C(t)$ represses its own transcription with a delay Δ. (F) Zwicker [35] model for coupled phosphorylation (PPC, purple background) and transcription-translation (TTC, blue background) cycles. KaiC hexamers switch between an active conformational state (circles) in which their phosphorylation level tends to rise and an inactive state (squares) in which it tends to fall. Active KaiC activates RpaA and inactive KaiC inactivates RpaA; active RpaA (red) activates *kaiBC* expression, leading (after a delay) to the injection of fully phosphorylated KaiC (pink) into the PPC.

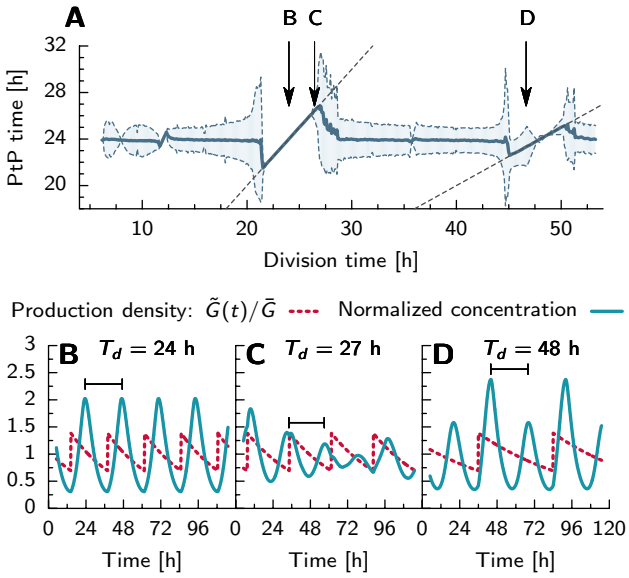

FIG. 2. Periodic gene replication dramatically affects a negative transcriptional feedback oscillator (NTFO). (A) The average peak-to-peak time $\langle T_{\rm PtP}\rangle$ (solid curve) versus the cell division time $T_{\rm d}$ at fixed $\mu_{\rm tot}$ and β. The shaded region shows the standard deviation of the peak-to-peak times (see *SI text*). Dashed lines indicate regions where the clock locks to the cell cycle with periods in a 1:1 (left) or 2:1 (right) ratio. (Smaller locking regions around $T_{\rm d} = 6, 12,$ and 36 h are not marked.) (B–D) Protein concentration $C(t)$ (blue solid line) and the protein production density $\tilde{G}(t) = g(t-\Delta)/V(t)$ (red dashed line) for the values of $T_{\rm d}$ indicated by the arrows in (A); horizontal brackets in (B–D) illustrate the definition of the peak-to-peak time $T_{\rm PtP}$. At $T_{\rm d} = 24$ h (B), the clock locks firmly to the cell cycle. For $T_{\rm d} = 27$ h (C), the cell-cycle period is just too large for locking; as a result, the cell cycle dramatically disrupts the clock, leading to a large standard deviation of $T_{\rm PtP}$ (see panel A). At $T_{\rm d} = 48$ h (D), two oscillation cycles of the NTFO fit exactly in one division time. The larger amplitude oscillation cycle corresponds to cell cycle phases where $\tilde{G}(t)$ is higher and the smaller amplitude to phases where $\tilde{G}(t)$ is lower. Similar results are obtained upon varying $T_{\rm d}$ at constant $\mu_{\rm act}$ (Fig. S3).

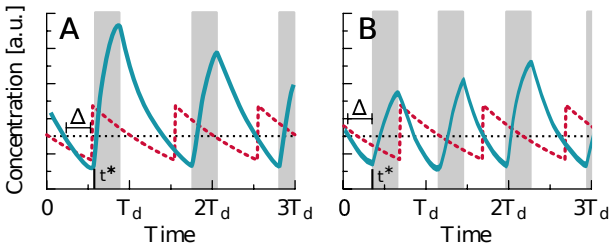

FIG. 3. Locking mechanism for the NTFO. Shown are time courses of the production density $\tilde{G}(t) = g(t-\Delta)/V(t)$ (dashed red lines) and the protein concentration $C(t)$ (solid blue lines). For clarity, we consider the limit $n \to \infty$, in which the Hill function describing autoregulation (Eq. 2) reduces to a step function with repression threshold K_c, denoted by the dotted horizontal line. Shaded regions indicate times when $C(t)$ is rising. The panels correspond to two different initial phase differences between the NTFO and the cell cycle. In each case, when $C(t)$ drops below K_c at time $t^* - \Delta$, protein production starts, but because of the delay Δ, new molecules are injected into the system only at time t^*. (A) The gene has replicated just before $t^* - \Delta$, and $\tilde{G}(t^*)$ is hence large, yielding a large amplitude for the next NTFO cycle. Because the rate of protein decay is independent of $\tilde{G}(t)$, the period of the NTFO cycle is correspondingly long. The subsequent NTFO cycle thus begins at smaller $\tilde{G}(t^*)$, causing it to have a smaller amplitude and a shorter period. (B) The gene has not yet replicated at time $t^* - \Delta$, and $\tilde{G}(t^*)$ is therefore low; consequently, the amplitude and period of the next NTFO cycle are small. The beginning of the subsequent cycle is then shifted towards higher $\tilde{G}(t^*)$, increasing its period. In both cases, the result is that, after a few cell cycles, the period of the NTFO oscillation approaches that of the cell cycle, yielding stable 1:1 locking where the two oscillators have a well-defined phase relation. The largest amplitude and thus longest possible clock period arise when the protein synthesis phase (grey bar) coincides with the maximal $\tilde{G}(t^*)$; if T_d increases beyond this maximal period, locking cannot occur. An analogous loss of locking occurs if T_d decreases below the minimal possible clock period. In either case, the clock shows erratic behavior until T_d approaches values where 1:2 or 2:1 locking is possible.

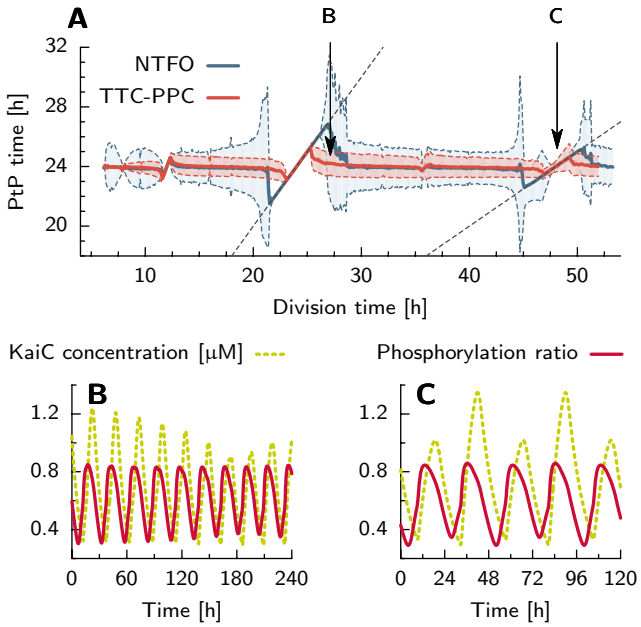

FIG. 4. A clock with interlocked phosphorylation and transcriptional cycles is more robust against perturbations from periodic gene replication. (A) The average peak-to-peak times $\langle T_{\mathrm{PtP}} \rangle$ of the phosphorylation level $p(t)$ of the coupled PPC-TTC model of the Kai system [35] (red solid curve) and of $C(t)$ of the NTFO (solid blue curve, same as Fig. 2A), as a function of the cell division time T_{d}. The shaded regions show the standard deviation of T_{PtP}. Both the widths of the locking regions and the standard deviations of the peak-to-peak time outside the locking regions are smaller for $p(t)$ of the Kai system than for $C(t)$ of the NTFO. Arrows indicate division times for which we show time traces in (B,C). (B) The total KaiC concentration $C_{\mathrm{tot}}(t)$ (dashed line) and $p(t)$ (solid line) at $T_{\mathrm{d}} = 26$ h. Though the amplitude of $C_{\mathrm{tot}}(t)$ is strongly affected by gene replication, the amplitude of $p(t)$ is nearly constant. (C) Plots of $p(t)$ and $C_{\mathrm{tot}}(t)$ at $T_{\mathrm{d}} = 48$ h, where the amplitude of $C_{\mathrm{tot}}(t)$ alternates between a low and a high value depending on the gene copy number in the cell. In contrast, $p(t)$ is almost unaffected by gene replication.

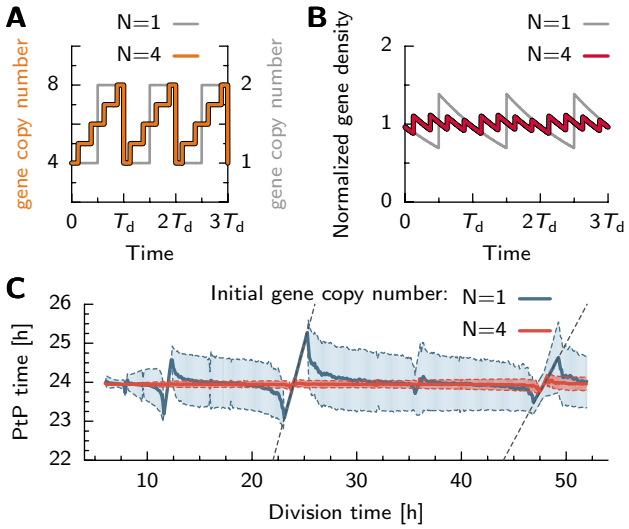

FIG. 5. A higher average gene copy number strongly reduces the effect of the cell cycle on the circadian clock. (A) Time course of the gene copy number $g(t)$ for initial gene copy numbers $N = 4$ (thick curve, left axis) and $N = 1$ (thin curve, right axis); time in units of cell cycle time $T_{\rm d}$. The increase in $g(t)$ is more gradual for $N = 4$ than for $N = 1$. (B) The gene density $G(t) = g(t)/V(t)$, normalized to its time average, for $N = 4$ (thick curve) and $N = 1$ (thin curve). At a higher gene copy number, the deviations from the average gene density become smaller. (C) The average peak-to-peak time $\langle T_{\rm PtP} \rangle$ of the phosphorylation fraction $p(t)$ of the PPC-TTC model of the Kai system [35], for initial gene copy numbers $N = 1$ (solid blue curve, same as Fig. 4A) and $N = 4$ (solid red curve) versus cell division time $T_{\rm d}$. (Note the y-axis range is smaller than in Fig. 2A and Fig. 4A.) For the higher gene copy number, the locking regions have almost disappeared and the standard deviation in the peak-to-peak times is very small. For time traces, see Fig. S5.