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Circadian clock effects on cellular proliferation: Insights from theory and experiments

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Abstract

Oscillations of the cellular circadian clock have emerged as an important regulator of many physiological processes, both in health and in disease. One such process, cellular proliferation, is being increasingly recognized to be affected by the circadian clock. Here, we review how a combination of experimental and theoretical work has furthered our understanding of the way circadian clocks couple to the cell cycle and play a role in tissue homeostasis and cancer. Finally, we discuss recently introduced methods for modeling coupling of clocks based on techniques from survival analysis and machine learning and highlight their potential importance for future studies.

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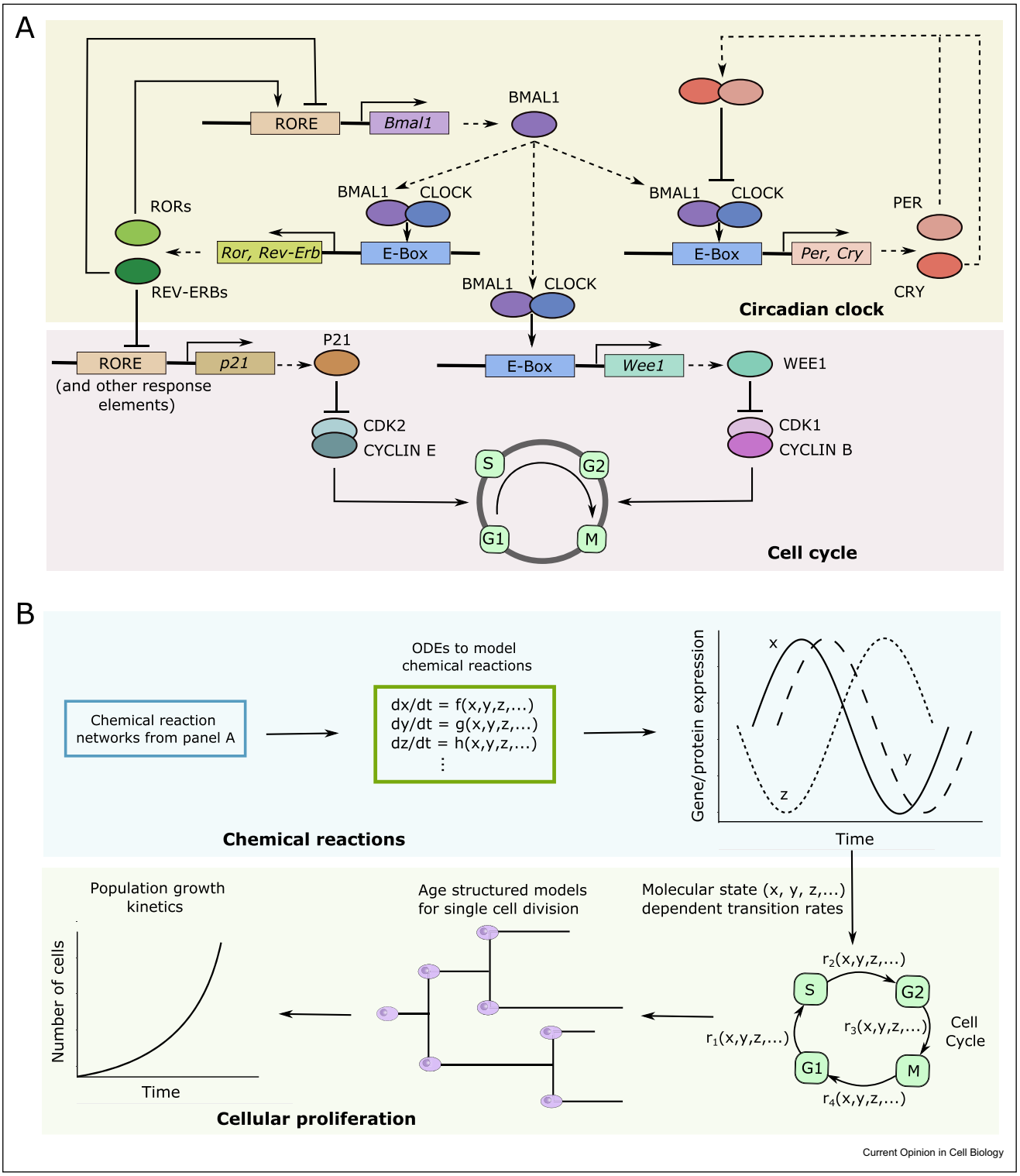
Introduction

Biochemical oscillations are ubiquitous in living organisms, arising from complex spatio–temporal interactions between genes, proteins and metabolites [1]. The circadian ('*circa*' — about, '*diem*' — day, in Latin) clock represents a special class of such biochemical oscillators; it has an intrinsic period of approximately 24 h and is thought to have evolved in organisms to allow anticipation of daily changes in the environment tied to the Earth's rotation [2,3]. These oscillations are self-sustained in single cells under constant environmental conditions and can be entrained by external cues such as light. It has become increasingly clear that the circadian clock plays an important role in regulating the cell cycle, thus affecting cellular proliferation in multiple contexts such as tissue homeostasis and cancer [4]. With a focus on the mammalian circadian clock, here we review recent progress in our understanding of the nature of coupling of the clock with the cell cycle. Recent reviews have summarized experimental evidence of this coupling and its potential consequences on human health [5,6], we provide a perspective on how a synergy between experimental and theoretical studies has led to significant insights in this rapidly growing field. We also discuss the potential usefulness of novel theoretical and computational approaches rooted in biostatistics and machine learning and, using a few recent studies as examples, discuss how such approaches in a data-rich age may prove invaluable for the future of circadian research.

Basic architecture of the mammalian circadian clock

Sustained oscillations in mammalian cells arise from a canonical set of interlocked transcriptional–translational feedback loops (TTFLs, Figure 1a), although the absolute necessity of transcription has been questioned, for example, from observations of circadian peroxiredoxin oscillations in human red blood cells which lack nuclei [7]. Heterodimers of the BMAL1 and CLOCK proteins bind Ebox motifs in the promoters of *Per2* and *Cry1*, leading to transcription and translation of the latter. PER2 and CRY1 proteins eventually are transported back into the nucleus, where they repress BMAL1 and CLOCK to decrease their own expression. Finally, degradation of PER2 and CRY1 over time allows BMAL1–CLOCK driven expression to switch back on, thereby establishing the circadian oscillation. In a second interconnected loop the BMAL1–CLOCK heterodimer induces transcription of *Ror* and *Rev-erb* genes, which in turn stimulate/inhibit *Bmal1* expression, respectively, by binding to ROR response elements (Figure 1a). We refer the interested reader to

Figure 1



Transcriptional–translational feedback loops (TTFLs) that generate circadian oscillations and coupling to the cell cycle. **(a)** The core feedback loops involving BMAL1, Clock, Per, Cry, Rev-Erb, and Ror are shown along with a few example modes of coupling of the clock to the cell cycle. The colored ovals represent proteins. **(b)** A schematic of some common approaches to mathematical modeling of the circadian clock and how it can drive cellular proliferation. The reactions from panel (a) are modeled using ordinary differential equations (ODEs), generating temporal dynamics of various components of the TTFLs [10–14]. These time-dependent molecular concentrations can then be used to define transition rates between various phases of the cell cycle, which can in turn be used in age-structured models to connect single-cell dynamics to population-level growth [25].

recent reviews providing detailed descriptions of the biochemical pathways involved [8,9]. The importance of these interconnected feedback loops in generating robust oscillations, and how the period and amplitude of the emergent oscillations are affected by genetic perturbations, have been studied in detail using mathematical models of these TTFLs [10–13] (Figure 1b). Fitting such models to gene expression data sets has also suggested tissue-specific differences in network motifs that underlie the essential feedback loops generating circadian oscillations [14].

Molecular mechanisms and mathematical models of circadian clock–cell cycle coupling

Early seminal studies demonstrated the existence of coupling between the canonical TTFL components of the circadian network and the cell cycle, revealing regulation of c-*Myc* transcription by *PER2* [15] and regulation of the G2/M inhibitor *Wee1* by BMAL1–CLOCK [16] (Figure 1a). Since then, a variety of molecular interactions between the two cellular oscillators have been uncovered [6]. Circadian modulation of the cell cycle is thought to occur primarily via coupling to the G1-S and G2-M transitions [6] (Figure 1a), although an earlier fate decision to enter G1 or G0 phases has also been suggested to be under circadian control in adult brain neurogenesis [17]. A number of modeling studies have predicted clock-controlled cell cycle entry: a model of BMAL1-driven enhancement of a CyclinD/Cdk4-6 inhibitor (posited to be p21) was able to explain enhanced cell proliferation after BMAL1 ablation in the subgranular zone of the adult hippocampus [17]. In another study, we investigated the possible origin of surprising intermitotic time correlations in colon cancer cell lineages, both in the absence and presence of the chemotherapeutic agent cisplatin. Our mathematical model predicted circadian control of cell cycle entry as an important regulator of cell cycle speed [18]. These models suggest early control of cell cycle progression by the circadian clock and point to an interesting avenue for further experimental and theoretical studies.

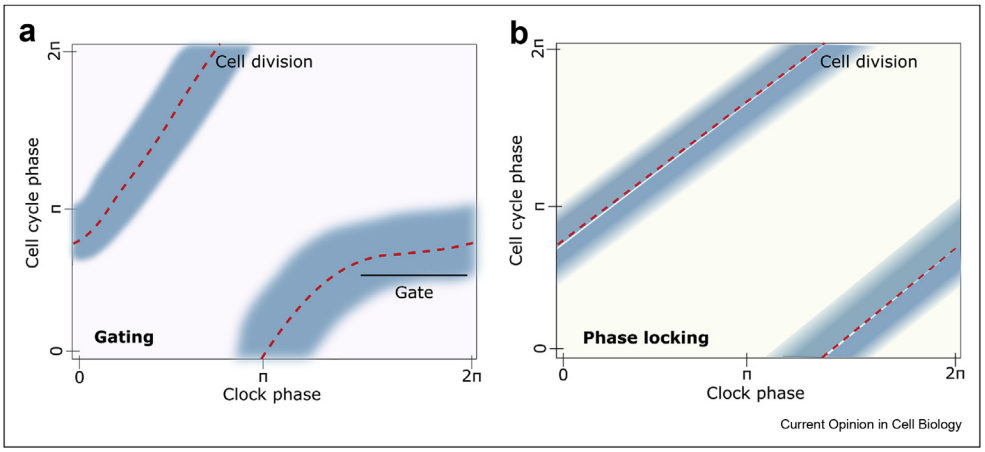
The G1/S transition has been demonstrated to be regulated by circadian control of phosphorylation of the retinoblastoma protein [19], WNT signaling [20], p21 [21], and p16 [22] (Figure 1a). The G2/M transition is affected by transcriptional regulation of *Wee1* by BMAL1–CLOCK [16]. The resulting circadian oscillations in WEE1 in turn regulates *Cyclin B1* expression, thereby allowing circadian control over the G2/M transition [23] (Figure 1a). Mathematical models investigating the consequences of these various modes of coupling have led to interesting and nonintuitive insights — for instance, it was shown that the domain of entrainment does not increase with increasing modes of clock and cell cycle coupling [24]. This result was based on the modeling prediction that the domain of entrainment via a combination of Wee1, p21, and cyclin E was not larger than the domain of entrainment through Wee1, p21, or cyclin E on their own. This interesting prediction suggests that perhaps the presence of multiple modes of coupling may provide redundancy rather than facilitating entrainment [24].

Although there are increasing reports of circadian driving of the cell cycle, relatively few studies have investigated the reverse coupling — modulation of the circadian clock by the cell cycle. An interesting combination of time-lapse microscopy and stochastic modeling of coupled oscillators provided strong evidence for a dominant reverse coupling in single mouse fibroblasts [26]. Using a maximum likelihood approach to infer the coupling function between the two oscillators, the authors found the strongest interaction to be an acceleration of the circadian phase right around the cell division event [26]. Although this work did not provide a mechanistic basis for the reverse coupling, more recent experiments have uncovered two modes of this regulation: (1) ubiquitination and subsequent degradation of Rev-erb-a is dependent on CDK-1 mediated phosphorylation of Rev-erb-a and controls the circadian oscillation amplitude [27] and (2) the transcription factor MYC disrupts the circadian clock in cancer cells by downregulating the core clock genes BMAL1 and CLOCK, either via upregulating Rev-erb-a [28] or by forming a complex with MIZ1 [29]. In turn, disruption of the clock affects cellular proliferation: upregulation of MYC attenuates the clock and promotes cell proliferation whereas its downregulation results in strengthening of the clock and reduction of cell proliferation [29]. Finally, the larger scale consequences of this reverse coupling (in addition to the previously discovered circadian clock to cell cycle coupling) were recently investigated using modeling; the authors predicted that bidirectional coupling results in more robust synchronization than unidirectional coupling [30].

Does the circadian clock 'gate' the cell cycle?

Although studies of the molecular mechanisms of clock–cell cycle coupling are relatively recent, reports of the preponderance of cell divisions at specific times of the day have existed since the early 1900s. A study of the dinoflagellate *Ceratium fusus* in the waters of the English Channel suggested that these unicellular organisms divide mostly between 1am–3.30 am [35]. Later studies of both unicellular eukaryotes [36] and prokaryotes [31] in culture showed that after entrainment to 12 h light and dark cycles, a large fraction of cell divisions occurred during a relatively short period of time around the late subjective night. These studies led to the use of the term 'circadian gating' (Figure 2a), which refers to the existence of circadian phases where cell cycle progression slows down or stops (gate closed)

Figure 2



Gating versus phase-locking modes of coupling between the circadian clock and the cell cycle. The two panels show schematics of phase portraits that can be obtained using time-lapse microscopy of single cells comprising circadian and/or cell cycle reporters. The red dashed curves represent average trajectories, and the blue zones denote the phase space through which typical cellular trajectories pass. **(a)** Gating is characterized by regions of phase space where cell cycle progression slows down or stops [31,32] **(b)** A 1:1 phase-locked state is depicted here, where the circadian clock and cell cycle progress in synchrony such that knowledge of the phase of one oscillator specifies the phase of the other to a large extent. Unlike in gating, the phase-locked state does not exhibit regions of significant cell cycle slow down [26,33,34].

and phases where the cell cycle progresses leading to cell division (gate open) [31,32].

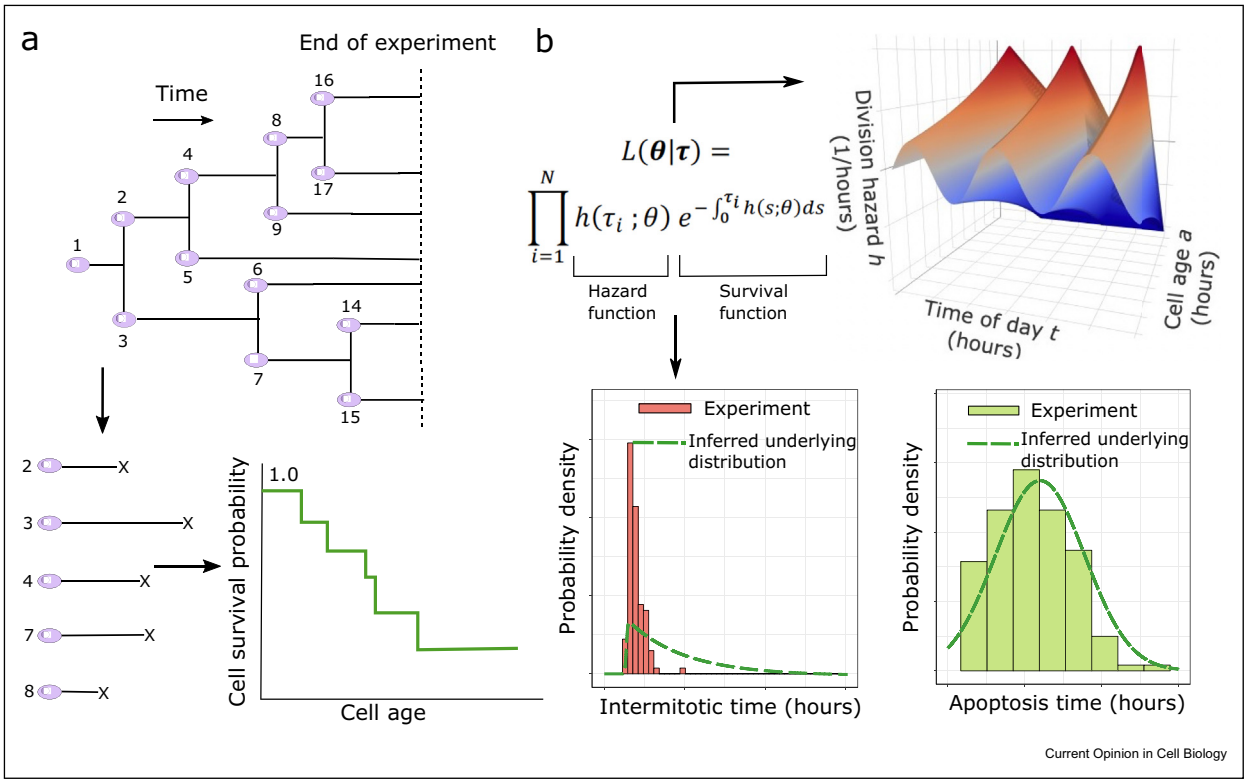
Phenomenological models of coupled oscillators combined with time-lapse microscopy of proliferating cells, tagged with fluorescent circadian and cell cycle reporters, are becoming increasingly important to infer the precise nature of the coupling between the circadian clock and the cell cycle. Such methods have recently suggested a need for going beyond the idea of gating to that of a more continuous, possibly bidirectional coupling between the clock and the cell cycle such that the two oscillators remain phase-locked [26,33,34] (Figure 2b). Other modeling approaches have also used the idea of a continuous modulation of cell division rates by the circadian clock to explore the origin of lineage correlations in intermitotic times [18,37,38], cell size control [39], and timing of cell divisions in bacteria [40]. However, a number of recent modeling efforts in 3D murine intestinal organoids [20] and in zebrafish [41] have suggested gating to be the predominant mode of coupling. In the zebrafish study, different light–dark (LD) cycles were imposed on a zebrafish-FUCCI cell line in culture. From the observation that the differing LD cycles made no difference to the average cell cycle length, while the number of mitosis events oscillated with time in all LD conditions, the authors suggested a gating mechanism over phase locking [40]. More studies will be necessary to elucidate the precise nature of the coupling in various organisms and cell types. Recent developments in generating endogenous reporters of the circadian clock using CRISPR knock ins will undoubtedly prove invaluable in this endeavor [42].

Use of survival analysis to model the circadian clock–cell cycle coupling

Nonlinear dynamical systems have been the most popular modeling approach for understanding how the circadian clock couples to the cell cycle [26,33,34]. Recently, we and others independently introduced methods from survival analysis to model the clock–cell cycle coupling [18,39]. Survival analysis is a set of statistical tools to analyze data where the variable of interest is time until an event occurs [43], for instance, in medical fields where time to death of patients is under study. The distinguishing factor of survival analysis is that it naturally deals with various scenarios of censoring, where the end of the observation period or other competing events precludes observation of the time to event for many individuals [44]. A basic introduction to survival and competing risks analysis in the context of single-cell time-lapse data is discussed in our recent work [18].

As shown in Figure 3a, the formalism of survival analysis lends itself naturally to the analysis of time-lapse microscopy data of proliferating cells. The central quantity is the hazard function h, which in this context is interpreted as the instantaneous rate of division of cells of a particular age a, given that the cells have survived until time a since birth (Figure 3b). The circadian clock can then be modeled as modulating the hazard function [18,39] and an analytic expression for the likelihood of observing a set of single-cell division times can be used for making inferences of underlying model parameters (Figure 3b top). In a recent work, this approach was combined with cubic B-splines to

Figure 3



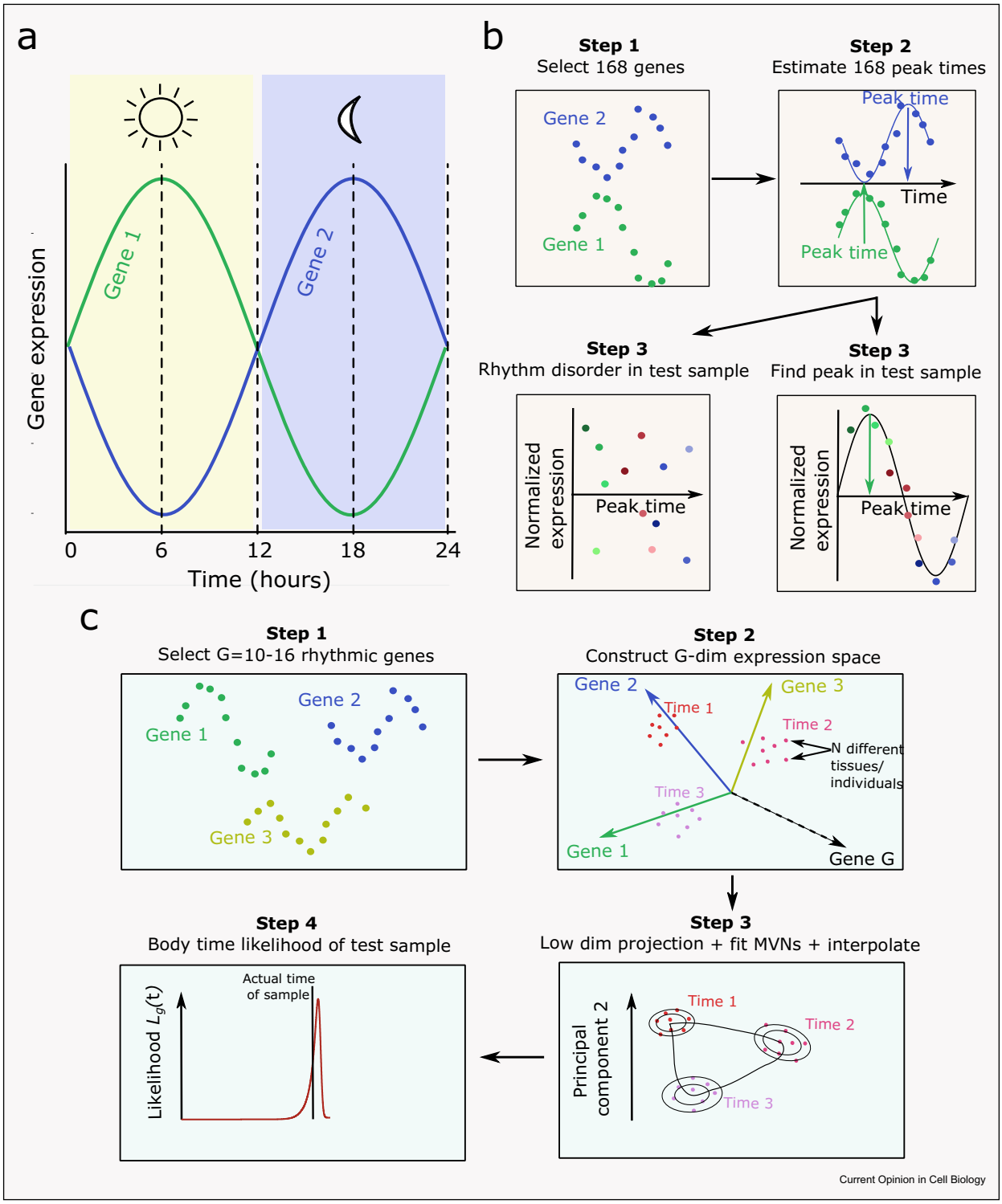
Survival analysis for modeling circadian coupling with the cell cycle. **(a)** A schematic showing how cellular proliferation can be mapped onto the problem of survival analysis, where every cell division is considered an 'event' and denoted by an 'x'. **(b)** A schematic of the basic approach to inference using survival analysis. The likelihood (L) of observing a data set comprising division (and/or apoptosis) times of all N cells (τ) is written down in terms of hazard functions. The underlying parameters (θ) are then inferred, often using Markov chain Monte Carlo methods [18,39]. The hazard for division can be modeled as a function of the circadian clock (or time of day t) and other covariates, and the parameters inferred computationally [39] (top). Red denotes larger hazard for division whereas blue represents lower hazard. In experiments performed in the presence of drugs, multiple cell fates such as cell division, death, and arrest can be induced (bottom). 'Competing risks' survival analysis can be used to infer the unbiased underlying division and apoptosis time distributions (bottom; green dashed lines) [18]. The experimentally observed distributions (bottom; red and green histograms) may be highly skewed and different from the underlying distributions depending on the drug concentration used.

flexibly model and infer how the circadian clock affects cell divisions in the cyanobacteria *Synechococcus elongatus* [39] (Figure 3b top). We combined a conceptually similar inference approach with the theory of copulas (that allow modeling of correlations in multivariate non-Gaussian distributions) to infer cell division and death times from correlated single-cell lineages [18]. Our method demonstrated how experimental observations of cell division times (red histogram; Figure 3b bottom) can be highly skewed in the presence of drugs [18], resulting in the underlying unbiased distribution becoming very different from the observed one (green dashed lines; Figure 3b bottom). Our computational approach paves a way for future studies to account for drug-induced biases while inferring the circadian clock–cell cycle coupling from time-lapse data. Furthermore, the survival analysis approach allows modeling of additional factors such as cell size [39] or delays in drug action [18], which may regulate cell division and affect inferences of the coupling function. Taken together, these studies show that survival analysis is a powerful tool for inferring effects of the circadian clock on the cell cycle.

Consequences of the circadian clock–cell cycle coupling in adult (cancer) stem cells

Although most cells across mammalian tissues are fully differentiated and hence postmitotic, adult stem cells make up a small but essential portion of tissues. These stem cells retain the capability of proliferating and generating new cells of the tissue, thus playing a critical role in tissue homeostasis, regeneration, and tumorigenesis. Understanding cell cycle control mechanisms in these stem cells is therefore essential, and regulation by the circadian clock has been demonstrated in adult skin, intestine, blood, hair, bone, and nerve stem cells [45]. Intriguingly, pluripotent stem cells do not exhibit circadian oscillations of the canonical TTFL genes [46],

Figure 4



Examples of methods that determine body/clock time or clock (a)synchronicity from single samples. **(a)** A schematic of the basic underlying principle behind determination of body/clock time. Different genes oscillate with fixed phase relationships with each other as well as external time in healthy individuals, allowing time to be inferred probabilistically from gene expression levels at a single time point. (b–c) Two distinct computational methods that use the basic principle in panel (a) to determine body time. **(b)** The molecular timetable method [60] uses genome wide expression levels and the peak times of a set of 168 oscillating genes to create a lookup table. These peak times are then used to determine the time of a test dataset. **(c)** TimeTeller [65] finds 10–16 oscillating genes (Step 1) to create a high dimensional representation of expression levels (Step 2). This data is then projected down to a lower dimensional space using a projection operator calculated from the N data points corresponding to one time point (Step 3). Separate multivariate

although noncanonical 24-h oscillations have been reported in metabolic programs of these cells [47].

Over the last few years, important connections between the circadian clock and cancer stem cells have emerged [4]. Traditionally, the circadian clock has been thought of as a tumor suppressor [48]. In support of this idea, B16 melanoma cells *in vitro* and tumors *in vivo* were found to suppress clock genes, and their proliferation was strongly reduced upon restoring clock function [49]. In addition, in support of the idea of circadian genes acting as tumor suppressors, an earlier study showed a direct protein−protein interaction between PER2 and the tumor suppressor P53; by forming a stable trimeric complex with P53 and P53's negative regulator MDM2, PER2 prevented ubiquitination of MDM2, and the resulting degradation of P53 [50]. Intriguingly however, recent studies have suggested the possibility of circadian genes aiding tumor maintenance in some contexts. For example, it was observed that while glioblastoma stem cells, differentiated glioblastoma cells, and noncancerous brain cultures exhibited circadian rhythms, only the glioblastoma stem cells showed a strong dependence on BMAL1 and Clock for optimal cell growth [51]. Similar effects were observed in hematopoietic cells, where *Clock* and *Bmal1* are required for leukemia cell growth in a murine model of acute myeloid leukemia, and circadian disruption impaired cell cycle progression [52]. Although these results are apparently contradictory and suggest a complex relationship between the circadian clock and cancer, theoretical modeling is well poised to shed light onto these complexities. For example, a study coupling ODEs to model chemical reactions and age-structured population models to describe population growth (Figure 1b) investigated the effects of *Per/Cry* mutations and *BMAL1* knockouts on cellular proliferation. This study concluded that depending on the autonomous period of the cell cycle (cell cycle length in the absence of coupling to the circadian clock) a disrupted circadian clock can lead to both enhancement and decrease of the cellular growth rate [25].

Circadian clock (de)synchronization: quantification and implications for cellular proliferation

A combination of experimental and theoretical approaches has provided fundamental insights into how individual cellular oscillators in mammalian tissues decode environmental information to stay synchronized [53−56] or become desynchronized by light perturbations, such as jet lag and similar protocols [57,58]. Many lines of evidence have suggested that synchrony among circadian clocks is crucial for maintaining healthy tissues, and desynchronization can lead to susceptibility to diseases such as cancer [4,59]. Using various tissues from mice exposed to chronic jet lag [60] and blood samples from humans undergoing a night shift protocol [61], it was observed that oscillations of circadian clock genes are dampened (presumably due to reduced synchrony among individual cells) and phase shifted. An *in vitro* jet lag−like protocol, developed to mimic these observations in cultured cancer cells [19], led to upregulation of cell cycle genes and a concomitant increase in cellular proliferation in human U2 osteosarcoma cells [19]. In mice, a jet-lag protocol was found to change expression levels of both tumor suppressor genes such as *NF1* and oncogenes such as *KRAS*, which in turn were associated with clock genes such as *Bmal1*, *Cry1*, and *Cry2* [62]. Cellular proliferation may therefore be linked to the degree of synchronicity among individual cellular circadian oscillators in a population of cells.

These results demonstrate the need to develop quantitative measures of clock (de)synchronicity in tissues of individual patients and investigate its association with disease, an endeavor to which computational modeling has made significant contributions over the years (Figure 4a). In early work, genome-wide expression patterns were used to infer body time [63] (Figure 4b). In more recent work, a variety of machine learning methods have been used to infer either body time or the degree of clock (a)synchronicity from single patient samples, often using a much reduced set of core clock genes [64−69]. For example, TimeTeller is a recently developed method which defines a metric for clock dysfunction from single samples based on the phase relationships between various circadian genes in diseased tissues compared with normal ones [68] (Figure 4c describes the TimeTeller workflow). Interestingly, this study demonstrated that the dysfunction metric was a prognostic factor for both disease-free survival and overall survival in primary breast cancer patients, independent of previously established prognostic factors such as the meta-PCNA gene signature [68]. Another study developed a 12 biomarker gene set from human epidermis [69], and used a previously developed method ZeitZeiger [65] to report circadian phase from single samples. This method performed well across body sites, age, sex, and detection platforms, which are essential elements for ease of clinical implementation [69]. Finally, a method to quantify relative coupling strength among individual cells has also been suggested based upon the idea that period

normals (MVNs) are fit to the data points at each time. Splines are then used to interpolate between the entries of the mean vectors and the covariance matrices to obtain an MVN likelihood function with time-dependent mean and covariance (Step 3). This procedure is repeated using projection operators based off every time point, and all the likelihood functions are combined to give the final likelihood $L_g(t)$. The peak of $L_g(t)$ gives the inferred body time of the test sample (Step 4).

and phase distributions in an ensemble of cells become narrower upon increasing coupling strength [70].

Conclusions and future directions

Much progress has been made in elucidating the basic principles of coupling of cellular circadian clocks, with each other as well as with other oscillators such as the cell cycle. Here, we have highlighted how a synergy between experiments and theoretical modeling has provided novel insights in this fast-growing field. Rapid developments in microscopy and image analysis techniques are allowing careful quantitative analyses of circadian clock coupling, and we believe that the next few years will see exciting developments in this area. The nature of circadian coupling with the cell cycle in diseases such as cancer and in response to drugs remains poorly understood, and in our opinion, represents an important avenue of future research. Novel theoretical approaches based on survival analysis [18,39] and machine learning [64–69] could provide important tools to analyze large quantities of data, from *in vitro* and *in vivo* as well as clinical studies. Such approaches could be coupled with evolutionary models of cancer progression that allow for time-dependent changes in cellular growth and death rates [71] to optimize treatment regimens accounting for the circadian behavior [72–74]. Optimizing treatment regimens based on the circadian clock remains a challenging frontier [75], and the combination of experimental and theoretical techniques will, no doubt, break important barriers in this endeavor.

Conflict of interest statement

Nothing declared.

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