Cyclin/Forkhead-mediated coordination of cyclin waves: an autonomous oscillator rationalizing the quantitative model of Cdk control for budding yeast

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INTRODUCTION
Timing is important to living organisms: many distinct processes need to occur at definite times relative to one another, i.e. in partial synchrony and with well-defined phase differences. Examples are found in heart function, tissue differentiation, sleep/wake cycles, and adaptive responses to external challenges. Failure in the timing of processes that together establish a physiological function may compromise the viability of living cells, or may make them escape from regulation, thereby compromising the viability of multicellular organisms. And indeed, temporal coordination of the events that regulate cellular proliferation is also pivotal to health.

The eukaryotic cell division cycle is one of the clearest examples of such processes. It ensures the consecutive and alternate execution of a number of distinct and incompatible processes (‘phases’), namely cell growth (G1 phase), DNA replication (S phase), chromosome segregation (G2 phase), cell division (M phase), and in many cases cell maintenance (G0 phase). The cell would succumb and transform or develop to disease if DNA replication and cell division would occur simultaneously with multiple, possibly incomplete rounds of replication or an imbalanced DNA segregation between consecutive cell divisions.

To maintain the separation between these processes, a regulatory mechanism must be employed by the cell such that incompatible processes do appear one after the other, in a periodic, unidirectional, and irreversible manner. Other processes can and should partly overlap, starting at different times but ending simultaneously. Thus, the cell division cycle is a strategic choice for studying the fundamental aspects of timing, because it relies on the clearly incompatible processes of genome duplication and cell division. To determine molecular mechanisms that prevent their fatal overlap, the regulatory networks that control the incompatible processes may be explored through systems biology.

Because of its critical role in guaranteeing survival, the cell cycle network has been conserved across species during evolution, ranging from a simple, unicellular yeast to multicellular, higher organisms such as humans and plants. The cell cycle can therefore be ideally studied in model organisms such as budding yeast.

Vital temporal coordination: keeping the incompatible separate through cyclin waves
The maintenance of strictly alternating cycles of genome duplication and cell division requires a regulator. Periodic waves of activity of dimeric enzymatic complexes, called cyclin-dependent kinases, represent the driving force behind cell cycle progression. These complexes are composed by a Cdk kinase – the catalytic subunit – and a differential pool of cyclins – the regulatory subunits. In the budding yeast *Saccharomyces cerevisiae*, activity of the Cdk1 kinase is modulated upon binding of nine distinct phase-specific cyclins, which are grouped in four subgroups. Cyclins confer the substrate specificity that allows Cdk1 to drive the cell cycle through a definite order (see refs. 5-7 and references therein). Successive, coordinated periodic oscillations of cyclin/Cdk1 activities ensure unidirectionality and timing of cell cycle progression: they must be activated for entry into S phase and passage through metaphase, and must be inactivated to allow cytokinesis, spindle breakdown and licensing of replication origins for new rounds of DNA synthesis. To complete these events, phase-specific cyclins are regulated to generate waves of

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cyclin/Cdk1 activity, a functional property of cell cycle control (Fig. 1a).

Sequential activation of cyclins by regulated transcription is crucial for the timing of cyclin/Cdk1 activities, which in turn are required for robust transcriptional oscillations by modulating the activity of various transcription factors\(^ {10-13}\). Four cyclin-associated waves of transcription occur throughout the cell cycle\(^ {14-16}\). \(\text{CLN1, CLN2, and CLN3}\) are essential for passing START at the G1/S transition\(^ {17}\). \(\text{CLB5 and CLB6}\) drive a timely and efficient DNA replication in S phase\(^ {18-21}\). \(\text{CLB3 and CLB4}\) are involved in DNA replication and mitotic spindle formation at the G2/M transition\(^ {22,23}\). \(\text{CLB1 and CLB2}\) are necessary for mitotic spindle elongation and mitotic exit\(^ {22,24}\).

Transcriptional mechanisms regulating the expression level of cyclin waves have been widely studied\(^ {8,17-13,25}\). Periodic activation of transcriptional activities normally restricted to the G1 phase occurs in cells lacking all six mitotic CLB genes\(^ {26,27}\); however, Cdk1 activity is essential to ensure the correct timing of gene transcription in G1 phase, thus to coordinate S-G2-M events with G1 events\(^ {26}\). To drive cell cycle-dependent gene expression, transcription factors must be cell cycle-regulated, and the activity of those controlling cyclin genes may be dependent on the Cdk1 activity promoted by an earlier transcriptional cyclin wave. Indeed, the cyclin/Cdk1 activity modulates transcription factors’ activity, and acts as their effector to trigger the ordered program of cyclin expression\(^ {14}\). Moreover, Cdk1 and transcription network activities

**Fig. 1 Waves of mitotic (Clb) cyclins throughout cell cycle progression.** a Qualitative description of alternating waves of expression of mitotic cyclin/Cdk1 complexes and of their stoichiometric inhibitor Cki throughout the cell cycle phases. In budding yeast: (i) Cki indicates Sic1 (black color), which is expressed maximally in G1 phase and at a low level in the other cell cycle phases; (ii) Cbk indicates mitotic cyclins: Clb5,6 (red color) trigger DNA replication in S phase; Clb3,4 (blue color) trigger completion of S phase and early mitotic events in G2 phase; Clb1,2 (green color) trigger late mitotic events and cell division in M phase. 

b Scheme of regulations connecting cyclin transcription and Clb/Cdk1 complexes through the Fkh2 transcription factor. The synchronization of Clb cyclins occurs in steps: (i) Clb5 promotes \(\text{CLB3}\) transcription (solid red line); (ii) Clb3 promotes \(\text{CLB2}\) transcription (solid blue line); (iii) Clb2 further promotes \(\text{CLB2}\) transcription through a Clb2-mediated positive feedback loop (solid green line) (adapted from Linke et al.\(^ {47}\)). Dashed colored arrows indicate regulations that may occur between Fkh2/Ndd1 and Clb cyclins, following the physical interactions that have been shown experimentally\(^ {37}\). For simplicity, Cdk1 has been omitted.
are coupled by feed-forward loops (FFLs) to convert periodic oscillations of Cdk activity in transcriptional response, ensuring the correct temporal order of cell cycle events.

Is there a mechanism that ensures robustness of cell cycle timing? It has been shown that such mechanism exists, with cyclin/Cdk1 activities contributing to the robustness of transcriptional oscillations. In fact, although Cdk1 appears not to be the main regulator of transcriptional oscillations, deletion of all mitotic, also called Clb, cyclins (clbΔ) results in a substantial delay of the timing of cell division. It has been therefore proposed that coupling a cyclin/Cdk1 oscillator and a transcriptional oscillator may regulate cell cycle progression, such that timing of cyclin expression is controlled by the transcriptional oscillator; in turn, cyclin/Cdk1 complexes can modulate the transcriptional oscillator by controlling its amplitude and period.

Thus, alternated waves of cyclins — thereby of cyclin/Cdk1 activities — and transcriptional events are coupled to tightly control cell cycle progression. This coupling allows to keep the incompatible processes of genome duplication and cell division separate. These incompatible processes are therefore required to begin and end in a well-defined alternating regimen.

The cyclin/Cdk–Forkhead axis coordinates waves of mitotic cyclins

Although waves of cyclin/Cdk1 activity are critical for cell cycle transitions, it is not fully understood how the temporal occurrence of successive cyclin waves is managed. Specifically, the precise molecular circuitry responsible for the coordination of waves of cyclins is not known. As it has been highlighted in the fundamental contribution to the cell cycle field written in 1998 by Mendenhall and Hodge, the transcriptional regulation of a number of cyclin genes is not known.

Efforts have been made to identify the transcriptional network governing phase-specific waves of gene expression, both experimentally (see refs. 12,13 and references therein) and computationally (see refs. 27,34–37 for some of the many developed methodologies). For example, clb5 and clb2, the more abundant cyclins within the Clb5/Clb6 and Clb1/Clb2 pairs, respectively, are known to be activated through transcriptional heterodimers: the former by the Mlu1 cell cycle box (MCB)-binding factor, MBF (Mbp1/Swi6 dimer); the latter by the Swi-five factor, SFF (Mcm1/Fkh2/Ndd1 trimer)12,13. The mechanism through which transcription of these mitotic CLB cyclin genes is controlled involves Cdk activities, however it lacks a comprehensive understanding.

Clb2/Cdk1 activity, main regulator of the timing of cell division, promotes transcription of the CLB2 gene itself by a positive feedback loop (PFL). The regulation occurs through phosphorylation of the Forkhead (Fkh) transcription factor Fkh2 which, together with its cognate Fkh1, promotes cell division by regulating the CLB2 cluster that drives the G2/M gene expression. Of note, this leads to the paradox that cell division would not initiate in absence of Clb2. Furthermore, CLB2 transcription is not induced in the absence of Clb1-4-associated kinase activities and Clb5/Cdk1 can phosphorylate Fkh2, suggesting Clb5 as the trigger of CLB2 transcription. Strikingly, Fkh2 has been shown to be phosphorylated in vivo in a cell cycle-dependent manner during the G2/M transition, therefore suggesting the potential involvement of other mitotic Clb/Cdk1 activities, besides Clb2/Cdk1, which may be important for the accumulation of Clb1 and Clb2.

Thus, it is not known whether or not Clb/Cdk1 activities – with the exception of Clb2/Cdk1 – are required for CLB2 transcription, and whether or not Clb/Cdk1 complexes directly regulate CLB2 promoter or make use of additional mechanisms – as pointed out earlier. In addition, already in 1998 Mendenhall and Hodge stressed that, among the missing details of the cyclin/Cdk-1 mediated transcription: ‘Not all of the dominoes have been identified; virtually nothing is known about the factors regulating CLB3 and CLB4 transcription, for example. Completing the identification and the characterization of the interrelationships among these factors remains a major challenge in this field’.

Altogether, identification of the interdependencies between Fkh and cyclin-associated kinase activities for a timely CLB2 expression, as well as of the factors regulating CLB3 and CLB4 transcription has therefore been a major challenge in the cell cycle field since the last two decades.

To shed new light on the dynamic coupling between Fkh and cyclin-associated Cdk1 activities, the former have been recently investigated as targets for the latter. Through a systems biology-driven investigation of the interconnection between these molecular players responsible for the timely coordination of DNA replication with cell division, the sequential order of waves of Clb cyclins was demonstrated to be achieved by mutual coordination of Clb/Cdk1 activities with Fkh-mediated transcriptional activity (Fig. 1b).

In detail, a minimal mathematical, kinetic model of Clb/Cdk1 activities – implemented through Ordinary Differential Equations (ODEs) – was generated that predicts a Clb/Cdk1-mediated regulation of an activator molecule responsible for the control of CLB3 transcription. This prediction was successfully validated experimentally, through identification of Fkh2 as pivotal molecule: Clb cyclin waves are synchronized by Fkh2, and a Clb/Cdk1-mediated regulation of Fkh2 modulates Clb cyclin expression through a FFE. Thus Clb/Cdk1 and Fkh2 mutually coordinate one another. Fkh2 specifically binds to the CLB3 promoter, and promotes CLB3 expression as well as the timely appearance of Clb3 protein level. Beside the known interactions with Clb2 (M-phase cyclin) and Clb5 (S-phase cyclin), Fkh2: (i) stably interacts with Clb3 (G2-phase cyclin); (ii) co-localizes with Clb3, but not with Clb2, in S phase; and (iii) is phosphorylated by Clb3/Cdk1, besides by Clb2/Cdk1 and Clb5/Cdk1. In addition, Fkh2 has been shown to affect the formation of the mitotic spindle – also Clb5 is involved –, thus suggesting that it might regulate Clb3 that is involved in this process.

Not all of the dominoes have been identified and their periodic activity is dependent on cell cycle-regulated recruitment of the coactivator Ndd1 at the S/G2 transition. Similarly to Clb/Cdk1 complexes, Ndd1 also exhibits dynamics of activation and deactivation: Ndd1 degradation, through the Anaphase-Promoting Complex/Cyclosome activated by Cdh1 (APC/C<sup>Cdh1</sup>), generates a feed-forward regulation that governs the timing of its accumulation at the G1/S transition (Fig. 2a).
Furthermore, similarly to Fkh2, Ndd1 activity is dependent by kinase activity, specifically of Clb5/Cdk1, Clb2/Cdk1, and Cdc55. Thus, multiple mitotic Clb/Cdk1 complexes may be able to phosphorylate Ndd1 to promote its association to Fkh2 at the CLB2 promoter. The recent results support this view, with (i) Ndd1 stably interacting with Clb3, (ii) the Fkh2/Ndd1 complex co-localizing with Clb3 in S phase; and (iii) Ndd1 (and Fkh2) being strongly enriched to the CLB3 promoter. These findings, together with the evidence that Ndd1 is phosphorylated by Clb3/Cdk1, indicate that the association between Ndd1 and Fkh2 oscillates throughout the cell cycle and correlates temporally with the transcriptional activation of CLB3 and CLB2.

Altogether, a novel design principle is uncovered, where Clb/Cdk1 kinases and Fkh2/Ndd1 transcription activities are interlocked to control gene regulation. Within this tie, the Fkh2/Ndd1 transcriptional complex may be regulated by multiple Clb/Cdk1 activities, to guarantee a timely cell division.
The emergent properties of cell division can be investigated and reproduced by modeling efforts, and minimal mathematical models have been recently developed to capture essential behaviors of cell cycle temporal dynamics. These models were inspired by the experimental evidence that cells carrying a single mitotic cyclin/Cdk complex are progressing through the cell cycle in mouse and fission yeast.

Through simulation of a minimal model of the mitotic Cib/Cdk1 regulation in budding yeast, the computer-based prediction of the transcriptional regulation embedded in the linear cascade of Cib cyclin activation – Cib5,6/Cdk1 activate Cib3,4/Cdk1, which in turn activate Cib1,2/Cdk1 (Cib5 → Cib3 → Cib2) – has been validated experimentally. The Fkh2 transcription factor was uncovered to control the temporal expression of mitotic Cib waves, with its activity being modulated by Cib/Cdk1 complexes throughout the cell cycle. This transcriptional regulation may be realized with Cib5/Cdk1 and Cib3/Cdk1 promoting Cib2 transcription by phosphorylating Fkh2, either through the transcriptionally mediated linear Cib cascade (Cib5 → Cib3 → Cib2), or through a FFL in which the linear cascade can play the main role (Fig. 2b). FFLs are ‘network motifs’ highly favored during the evolution of transcriptional regulatory networks in budding yeast, and are described by three genes coding for transcription factors, say X, Y, and Z, with X and Y directly regulating Z and X regulating Y. The three regulations that occur among these three components can be activatory or inhibitory.

In an ‘incoherent’ FFL, the signs of the direct regulation – from X to Z – is opposite than the overall sign of the indirect regulation – from X to Z through Y. The ‘incoherent’ FFL generates pulses and accelerates or delays responses and, for this reason, it is referred to as ‘sign-sensitive accelerator’. In a ‘coherent’ FFL, the sign of the direct regulation – from X to Z – is the same as the overall sign of the indirect regulation – from X to Z through Y. The ‘coherent’ FFL may serve as a sign-sensitive delay element: a short pulse of the indirect regulation may occur either through a linear cascade (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs (Cib5 → Cib3 → Cib2) or through a coherent FFL in which the linear cascade has possibly a major role, either aided by Cib2/Cdk1-mediated PFLs 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able to exhibit cyclic Clb oscillations in a Boolean type of modeling effort.

This result did stimulate a deeper investigation about the designs responsible for autonomous cell cycle oscillations in budding yeast. Specifically, the number and nature of sets of motifs that were able to synchronize the oscillatory-like behavior of Clb/Cdk1 waves, thus reflecting the alternation of DNA replication and cell division, have been explored. Practically, network motifs that characterize autonomous oscillators were investigated by applying the System Design Space (SDS) methodology. This methodology relates genotype (in terms of the parameter values) to phenotype (referring to the combination of dominant reactions that may define a particular trait) by deconstructing a biochemical system into a finite number of qualitatively distinct network structures. Computationally, this translates into the following: for a given set of parameters and concentrations of model species, there exists a single ‘dominant positive term’, i.e. largest, and a single ‘dominant negative term’ in each ordinary differential equation (ODE). When reducing the mathematical description of the phenotypes to these dominant reactions, boundaries in the parameter space where the phenotype is valid may be obtained, and phenotypes may be sampled for oscillations.

This methodology has been employed to explore the areas in the full parameter and reaction state space where particular network structures (phenotypes) are prevalent (dominant) in the experimentally validated minimal model of Clb/Cdk1 regulation (see Linke et al. and reference therein). Through this analysis, which was improved by adding a search for complex conjugate eigenvalues with positive real part expected around Hopf bifurcations – from which limit cycles may arise – a search was conducted to explore whether, and to which extent, alternative motifs exist that contribute to the temporal and sustained coordination of Clb/Cdk1 complexes. Autonomous oscillations (also referred to as ‘limit cycles’) capturing the sequential activation and inactivation of waves of the three mitotic Clb/Cdk1 complexes and their stoichiometric inhibitor – Sic1 (G1 phase), Clb2,6/Cdk1 (S phase), Clb3,4/Cdk1 (G2 phase), and Clb1,2/Cdk1 (M phase) – were observed. A definite regulatory mechanism was identified that incorporates Clb3/Cdk1-centered regulations that self-sustain Clb/Cdk1 and Sic1 oscillations: a Clb3/Cdk1-mediated PFL, and the linear cascade of activation of mitotic Clb/Cdk1 complexes through Clb3/Cdk1 (Fig. 2d, e).

Specifically, the activatory regulations Clb3 → Clb3 and Clb3 → Clb2, forming the recently discovered Fkh2-mediated linear Clb cascade (Fig. 2d, e, solid black arrows), were found to be more frequently dominant in network designs that yield autonomous Clb/Cdk1 oscillations as compared to the Clb5 → Clb2 regulation described earlier (Fig. 2c, dotted black line). Moreover, a PFL mediated by Clb3/Cdk1 on Clb3 synthesis (Clb3 PFL) – or by Clb2/Cdk1 on Clb2 synthesis (Clb2 PFL) in absence of Clb3 PFL – improved the ability of the minimal model to generate sustained Clb/Cdk1 oscillations (Fig. 2d, e, solid red arrows), in agreement with early in silico analyses that predicted the ability of the Clb3 PFL to timely shape certain Clb waves, and with the hypothesis that the FFL - PFL structure underlies a well-timed cell division. Furthermore, with respect to the inhibitory regulations, the Clb2/Cdk1-mediated APC/C NFLs on Clb5/Cdk1 and Clb3/Cdk1 were more frequently dominant in network designs that yielded autonomous Clb/Cdk1 oscillations (Fig. 2d, e, bar-headed black lines).

In summary, in budding yeast, the Fkh2/Clb3 axis underlies autonomous oscillations of Clb/Cdk1 activities and their mutual coordination with Fkh2 transcriptional activity. Of note, a design in common between the network structures that exhibit oscillations can be observed: two activatory regulations through Fkh2 (Clb3 → Clb2 and Clb3 → Clb3) and one inhibitory regulation through APC/C (Clb3 → Clb2) (Fig. 2f). A PFL mediated by Clb3/Cdk1 on Clb3 synthesis (Clb3 PFL), improves the ability of the models to generate sustained Clb/Cdk1 autonomous oscillations. This design has been recently named ‘negative feedback with positive feedback loop’ (NF-PFL), and is a robust oscillator. Because Fkh2 is conserved across yeast species, including fission yeast and Candida albicans, and in filamentous fungi, it can be speculated that a network where Fkh2 modulates, and is modulated by cyclin/Cdk complexes may be involved in the order in time of incompatible cell cycle processes.

Outlook 1: CHECKPOINT versus AUTONOMOUS OSCILLATOR models of cell cycle control

Time-dependent responses of biological networks, such those occurring in the cell division cycle, may be accompanied by oscillatory behavior of their components, to convert stimuli to physiological output at a proper timing. A deregulation of this timing, thus of the staggered cyclin/Cdk oscillations that respond to extra- and intra-cellular signals, may impact on the coordination of the incompatible processes of DNA replication and cell division. Therefore, network designs (motifs) that exhibit timely oscillations are inherently crucial to sustain organismal physiology.

In budding yeast, cell cycle networks are typically modeled through the CHECKPOINT logic, which explicitly considers irreversible transitions between cell cycle states. In these models, developed by Tyson and Novak, the starting point of the simulations is reset upon reaching specific concentration thresholds of Clb5 for the onset into S phase, and for Clb2 for the onset into G2/M phase. Experimentally, these simulations correspond to scenarios where DNA damage/errors would activate the checkpoint affecting Clb5 levels – thus slowing/halting DNA replication dynamics – and where troubles in completing mitotic events would activate the checkpoint affecting Clb2 levels – thus delaying/impairing cell division. Therefore, in these models, oscillations may not be autonomous. Network motifs such as PFLs or NFLs – and their combinations – are able to generate oscillations in budding yeast, and the cell cycle may have been designed by evolution to oscillate, e.g. when no checkpoint needs to be activated. Therefore, it is remarkable that the AUTONOMOUS OSCILLATOR logic has not been shown yet for the available wild type yeast CHECKPOINT models.

There may seem to be a fine line between the CHECKPOINT and AUTONOMOUS OSCILLATOR views, which may have resulted in a conceptual misinterpretation for more than two decades. A few published cell cycle models may look as if they are autonomously oscillating; however, looking at how they have been implemented mathematically, they are not. Two examples of the CHECKPOINT logic considered by Tyson/Novak may be examined where seemingly autonomous oscillations are shown for a cell cycle model of fission yeast (indicated below as the 1997 model) and for a generic model of eukaryotic cell cycle regulation (indicated below as the 2006 model), differently from the AUTONOMOUS OSCILLATOR logic considered in cell cycle models by Goldbeter in mammalian cells and for Ferrell in Xenopus laevis, and by Barberis in budding yeast, where autonomous oscillations are found for minimal to medium-size models of the cyclin/Cdk network.

The 1997 model does contain checkpoints of the following type: (i) when SPF crosses 0.1 from below, S phase is initiated (Start); (ii) when Ube crosses 0.1 from above, the cell divides functionally (i.e. mass is divided by 2); and (iii) 60 min after Start, k0 is divided by 2, and at cell division k0 is multiplied by 2 (see Table 1 of that work for details). These rules indicate that, while the model is running, there are points in model time where variables are reset (e.g. mass, which directly affects rates in the system) or where parameter values are changed (k0). This means that, as the model is running it is forced by the events to jump non-continuously through the state space and parameter space. This is a significant difference.
compared to autonomous oscillatory models, among which the minimal model of the mitotic cyclin/Cdk1 network of budding yeast discussed here (indicated below as the 2020 model)\textsuperscript{95}, which contain neither of these jumps, and do not reset. As a consequence of such parameter and state space jumps and enforced oscillations in mass and parameters, in the 1997 model\textsuperscript{94} an increased likelihood of observing oscillations (either transient or permanent) exists. Similarly to the 1997 model, the 2006 model\textsuperscript{99} uses the same implementation of the mass variable and mass-dependent checkpoints, i.e. division (mass = mass/2), when actCyc8 decreases to 0.1 (fission yeast), 0.2 (budding yeast), and 0.3 (mammalian cell).

A difference between the 1997 and 2006 models by Tyson/Novák is that the 2006 model only uses a checkpoint that affects a single variable (mass). The mass then indirectly affects the other model variables by adjusting rates in the system, but it does not alter parameters during model runtime as it instead occurs in the 1997 model. In that sense, the 2006 model is closer to the approach shown for the 2020 model by Barberis, but still not quite the same because there are enforced state space jumps which do not occur in the latter. An interesting way of thinking about the difference between the 2006 and 2020 models is that, in the 2020 model, once the model starts to evolve in time and relaxes to a limit cycle oscillator it will permanently remain in the path determined by the limit cycle. Conversely, in the 2006 model, at specific points during the time course when the division event is triggered, the model is forced out from its current state into another state (mass = mass/2). This new state may or may not be in the attractor region where it was before. Of note, the steady state stability properties should remain the same, since no parameters were changed. In the 1997 model, the jump is even more severe due to the parameter changes that are performed, which may induce changes in the presence of, and stability of steady states, e.g. it may induce a bifurcation. The approaches shown for the 1997 and 2006 models are interesting from a computational point of view, but different from the approach considered in the 2020 model.

Of note, in the 2006 paper, it is reported that the bifurcation points, including the important SNIPER, occurs at a fixed mass. In all checkpoint models of the cell cycle, bifurcation analysis is performed by fixing the mass variable and turning it into a parameter. As a consequence, the model being integrated is then different than a model where the mass is a dynamic variable. This can also be observed in the 1997 model. Under such circumstances, the CHECKPOINT logic may reduce to something similar to the AUTONOMOUS OSCILLATOR logic of the 2020 model, given that there are no other checkpoints left in the model. importantly, the 2020 model structure was not designed to yield oscillations in general, but it can yield oscillations. Specifically, the 2020 model shows that Clb3-centered interactions are the network motifs underlying mechanisms of these oscillations\textsuperscript{25} that have been proven to exist in budding yeast cells\textsuperscript{17}.

The prediction that Clb3-centered regulations are the highest represented network motifs that drive autonomous oscillations in a minimal model of cyclin/Cdk control\textsuperscript{95} provides a possible ground to reconcile CHECKPOINT and AUTONOMOUS OSCILLATORY views. In a view of a dynamic cell cycle, autonomous oscillations driven by Clb3, thus by the Clb3/Cdk1 kinase complex, may occur when a coupling to the S and M phase kinase complexes is realized through a series of ‘clocks’ which coordinate together: Clb5 (CLOCK1), Clb3 (CLOCK2), and Clb2 (CLOCK3)\textsuperscript{102}. A recent mechanism has been proposed where ‘clock units’ control waves of Cdk activities, and therewith the temporal coordination of Clb/Cdk1 complexes: CLOCKS (Clb cyclins), DRIVER (Cdk1 kinase), TIMER (Sic1 inhibitor), CONTROLLER (Fkh2 transcription factor), and MODULATOR (Sir2, histone deacetylase)\textsuperscript{102}. Within these ‘clock units’, Clb5 and Clb2 respond to the checkpoint mechanisms (Tyson/Novák view), and Clb3 drives autonomous cell cycle oscillations coordinating Clb5 and Clb2 (Barberis view) to maintain cell proliferation. Thus, being Clb3 tightly coordinated with Clb5 and Clb2, an autonomous oscillator may be maintained (through Clb3-centered regulations) until the action of a checkpoint (through Clb5 and/or Clb2), which activation would then terminate the autonomous oscillations. This view provides a possible solution to the conceptual misinterpretation between CHECKPOINT and AUTONOMOUS OSCILLATORY logics, reconciling these views.

Clb cyclins, thereby Clb/Cdk1 activities, may be coordinated through a ‘reader-writer’ mechanism proposed for enzymatic gear shifters that exhibit a double functionality, one by which they ‘read’ the cell’s state and one by which they ‘write’ and modulate that state\textsuperscript{23}. In this sense, Cdk1 ‘writes’ by phosphorylating target proteins upon the binding of ‘readers’ Clb cyclins, or ‘clock units’, that activate Cdk1 by determining which of the possible protein substrates it will phosphorylate, thus determining Cdk1 specificity. ‘Readers’ and ‘writer’ operate in a quasi-independent manner in real time: as the cell cycle is running, Cdk1 first associates with one, then with a second, and then with the subsequent cyclins, to generate the characteristic waves of cyclins pattern over time. Within this scenario, the CHECKPOINT and AUTONOMOUS OSCILLATORY logics are reconciled through a ‘readers’–centered gear shifter mechanism (see Fig. 3 and its description in the accompanying figure legend) that sets in motion the activatory (Clb/Fkh2-mediated) and inhibitory (APC/C-mediated) regulations coordinating Clb waves (Fig. 2e).

After three decades from the pioneer in silico studies of Goldbeter and Tyson, who did show that a progressive activation and inactivation of a single cyclin/Cdk complex is able to generate its sustained oscillations\textsuperscript{104,105}, a minimal autonomous cell cycle oscillator independent of checkpoints mechanisms is discovered – for the first time – for budding yeast. It cycles by itself without any periodic reset, exhibiting sustained oscillations of mitotic Clb/Cdk1 complexes through a progressive accumulation of cyclin levels – thereby progressive activation of cyclin/Cdk complexes from S-to-M phase – to ensure unidirectionality of cell cycle progression. In principle, this scenario reflects the logic of the quantitative model of Cdk control that has been envisioned by the Nobel Prize 2001 recipient Sir Paul Nurse in 1996. This model proposes that a progressive cyclin accumulation leads to an increase in the Cdk activity through different thresholds of activity, with different thresholds of cyclin-mediated Cdk activity dictating progression through S phase and M phase\textsuperscript{106,107}. The molecularity underlying Sir Nurse’s quantitative model of Cdk control is currently not fully revealed, and the novel design principle proposed here may fill this gap in the knowledge for budding yeast; through exploration of the recently proposed ‘clock unit’ mechanism underlying the waves of cyclins pattern, where a progressive Fkh2 activation may be realized by the action of multiple Clb/Cdk1 complexes\textsuperscript{102}.

**Outlook 2: Role of cyclins for a well-timed cell cycle progression**

Because well-timed DNA replication and cell division maintain a healthy offspring, timely functioning of events driven by cyclins is ensured by their partially overlapping activities\textsuperscript{5,75}. For example, this overlap guarantees DNA replication to take place at a correct timing\textsuperscript{108}. Clb6 and Clb5 are partially overlapping in the regulation of early and late DNA replication, respectively, with Clb5 being the main regulator of the process, replacing Clb6 function in clb6Δ cells. In clb5Δ cells, S phase is prolonged\textsuperscript{21}, and the correct replication timing may be restored progressively after Clb2 activation\textsuperscript{108}. Conversely, in clb2Δ cells, defects in mitotic entry and delay in mitotic exit are observed\textsuperscript{23} because Clb5 or Clb3 cannot replace the missing Clb2 activity. Differently from Clb5 and Clb2, which deletions impact on the cell division timing, Clb3 deletion does not affect cell cycle timing\textsuperscript{109}. In fact, in clb3Δ cells,
cell division occurs at a correct timing because Clb2 can replace Clb3 activity. However, Clb3 deletion leads to an altered dynamic of cell division and is lethal in the clb2Δ clb3Δ double mutant\textsuperscript{19}, and in the clb5Δ clb3Δ clb4Δ\textsuperscript{19} and clb2Δ clb3Δ clb4Δ\textsuperscript{2,23,110,111} triple mutants. In these scenarios, Clb5 and Clb2 replace Clb3 (and Clb4) activity required for mitotic events such as spindle formation. Altogether, this bulk of evidence indicate that overlapping of waves of cyclins is instrumental to guarantee a correct timing of cell division.

Interestingly, Clb3 appears to be not evolutionarily conserved. Yet, this potential, yet uncovered, function of this mitotic cyclin highlights that the role of a protein can change through evolution across species, so the non-conservation of Clb3 may indicate a specificity of function in an organism, but not in another.
For example, the budding yeast at usual growth rates appears to operate exclusively in the limit cycle domain, whereas the fission yeast operates mostly in a stable steady state domain. Thus, it seems likely that any molecule, e.g. Clb3 in budding yeast, is more important for some organisms than for others. Furthermore, the lack of a phenotype of a clbΔ mutant suggests that the potentially less relevant genetic outcome for a gene deletion may hide a more sophisticated biochemical mechanism of regulation.47

Clb5, critical for the activation of DNA replication, is not essential in terms of survival because Clb2 can take over its role in clb5Δ cells.109 However, Clb5 is considered in all computational models of the yeast cell cycle because of the experimental evidence of its role. Therefore, it is not surprising that also Clb3 is not an essential gene, with its function being taken over by Clb2 in clb2Δ cells, as mentioned above (see Pecani and Cross109 and details in Mondeel et al.85). Similarly, also Fkh2 is not an essential gene, with its function being partially taken over by Fkh183,112,113, which may be involved in the regulation of Clb/Cdk1 activities47 although the molecular details of this mechanism are currently unknown.

Clb3 is lacking in existing computational models of the yeast cell cycle85,92, likely due to less explored functions as compared to those, well-known, of Clb5 and Clb2. However, although modulation of Clb3 activity is not required for mitotic exit89, its mitotic degradation is required for control of Start in G1 phase of the cell cycle. Strikingly, without mitotic destruction, Clb3 synthesized in the preceding cell cycle may directly activate Start, bypassing the requirement for the G1 (Cln) cyclins.109 This evidence, together with the discovery of the role that Clb3 has in the formation of the waves of cyclins pattern47, has resulted in the inclusion of Clb3 in recent computational models of the yeast cell cycle47,85. Based on simulation results through a solid methodology85, Clb3 may be important for the robustness of autonomous limit cycle oscillations of three Clb/Cdk1 kinase complexes and of their stoichiometric inhibitor Sic1, where all three pairs of cyclins – including Clb3 – exhibit oscillations22,23.

After two decades of scientific gap regarding the role of Clb3, its critical role in the occurrence of the waves of cyclins pattern is now supported by both experimental47 and computational85 analyses, which have unraveled a molecular design that involves a Forkhead molecule85. Within this design, network structures incorporating elements of FFL + PFLs regulations (Fig. 2c) may be involved for the occurrence of Clb/Cdk1 oscillations, with a relevance for the NF-PFL motif for their autonomous pattern.86

Altogether, a novel design principle actuating the quantitative model of Cdk control for budding yeast is proposed that may modulate the timing of cell cycle dynamics102. Given the evolutionary conservation of the cell cycle core machinery, this design principle of cellular proliferation that relies on cyclin/Cdk and transcription activities being interlocked may be envisioned in higher eukaryotes such as humans. Specifically, a core ‘clock unit’ incorporating this design has been recently proposed to be in place for both budding yeast and mammals102. It has a DRIVER (Cdk) operating its functions through multiple CLOCKS (mitotic Clb cyclins), with TIMERS (stoichiometric inhibitors of Clbs) determining whether and when the clocks are active, and CONTROLLERS (transcription factors) determining how quickly the clocks shall be active depending on external MODULATORS (e.g. epigenetic regulators)102. This ‘clock unit’ may coordinate temporal waves of cyclin/Cdk concentration/activity in the eukaryotic cell cycle, to keep the incompatible processes of genome duplication and cell division separated in time, thereby ensuring a robust and well-timed cell division.

DATA AVAILABILITY

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Received: 15 December 2020; Accepted: 1 November 2021; Published online: 13 December 2021

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**ACKNOWLEDGEMENTS**

M.B. would like to thank Francesc Posas, Jens Nielsen, Hans Westerhoff, Ioannis Xenarios, and Edda Klipp for their scientific support; Mart Loog for the availability to host some experiments in his laboratory; Christian Linke, Alberto González-Novoa, Silvia Tognetti, and Isa Al Sawad for their help with experimental settings; Thierry Mondeel, Anastasia Chasapi, Wolfram Liebermeister, and Oleksandr Ivanov for their initial drafting of Fig. 3; Paul Verbruggen for help with the final layout of the figures. This work was supported from the Systems Biology Grant of the University of Surrey. The corresponding author can also be contacted at matteo.barberislab.com.

**AUTHOR CONTRIBUTIONS**

M.B. conceived and formulated the ideas and hypotheses, designed the computational and experimental investigations underlying the studies described, and wrote the manuscript.

**COMPETING INTERESTS**

The author declares no competing interests.

**ADDITIONAL INFORMATION**

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Published in partnership with the Systems Biology Institute