A Clb/Cdk1-mediated regulation of Fkh2 synchronizes CLB expression in the budding yeast cell cycle

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Precise timing of cell division is achieved by coupling waves of cyclin-dependent kinase (Cdk) activity with a transcriptional oscillator throughout cell cycle progression. Although details of transcription of cyclin genes are known, it is unclear which is the transcriptional cascade that modulates their expression in a timely fashion. Here, we demonstrate that a Clb/Cdk1-mediated regulation of the Fkh2 transcription factor synchronizes the temporal mitotic CLB expression in budding yeast. A simplified kinetic model of the cyclin/Cdk network predicts a linear cascade where a Clb/Cdk1-mediated regulation of an activator molecule drives CLB3 and CLB2 expression. Experimental validation highlights Fkh2 as modulator of CLB3 transcript levels, besides its role in regulating CLB2 expression. A Boolean model based on the minimal number of interactions needed to capture the information flow of the Clb/Cdk1 network supports the role of an activator molecule in the sequential activation, and oscillatory behavior, of mitotic Clb cyclins. This work illustrates how transcription and phosphorylation networks can be coupled by a Clb/Cdk1-mediated regulation that synchronizes them.

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INTRODUCTION

In budding yeast, coordination of cell cycle transitions is achieved by periodic changes in the activity of the kinase Cdk1, which is regulated by temporal waves of expression of phase-specific cyclins.1, 2 Four waves of cell cycle-dependent transcription of cyclins are recognized: a wave of G1 cyclins (Cln1-3) is essential for passing START at the G1/S transition, whereas three waves of B-type cyclins (Clb5,6, Clb3,4, and Clb1,2) control DNA replication dynamics and mitotic entry/exit through S-to-M phases.3, 4 Successive oscillations of cyclin/Cdk1 activities ensure unidirectionality and correct timing of cell cycle transcriptional regulation. Although the yeast cell cycle has been studied extensively, it is not yet fully understood how these kinase activities interconnect precisely with the various transcriptional mechanisms driving a timely cell cycle progression. Cdk1 is not the main regulator of transcriptional oscillations,5 however, cyclin/Cdk1 activity contributes to the robustness of transcriptional oscillations by (i) modulating the activity of transcription factors, and (ii) acting as their effector to trigger the ordered program of cyclin expression.6 Moreover, transcription network and Cdk1-driven phosphorylation events are coupled by feed-forward loops to convert periodic oscillations of Cdk activity in transcriptional response.7 This ensures that the precise temporal sequence of cell cycle events is maintained.

A precise knowledge of the axis cyclin/Cdk1-transcriptional regulation throughout all cell cycle phases is still lacking. However, transcriptional regulation of the CLB2 cluster that drives G2/M gene expression has been widely investigated, and the forhead (Fkh) transcription factors Fkh1 and Fkh2 were identified as essential in this process.8, 9 Fkh1 and Fkh2 transcripts display a peak in S phase, and their periodic activity is dependent on cell cycle-regulated recruitment of the coactivator Ndd1 at the S/G2 transition.8 Although Fkh1 and Fkh2 have overlapping functions, only Fkh2 can associate with Ndd1 to regulate CLB2 expression.9 Fkh2 and Ndd1 are phosphorylated by Clb5/Cdk1, thus triggering recruitment of Ndd1 to Fkh2 to CLB2 promoter, and subsequently by Clb2/Cdk1 that further stabilizes the Fkh2-Ndd1 interaction by a positive feedback loop.10 Fkh2 phosphorylation is not abolished in a clb5Δ strain, suggesting that other Clb/Cdk1 complexes may also play a role in Fkh2 activation. Remarkably, in the absence of Clb3, Clb4, and Clb5, the CLB2 promoter is not fully active with Clb2 being highly unstable.11 Thus, multiple Clb/Cdk1 complexes could phosphorylate Fkh2 and/or Ndd1 to generate a basal level of CLB2 expression that would then rapidly increase due to the Clb2/Cdk1-mediated positive feedback loop.

Although many details of the transcription of cyclin genes are known,9 there is still a lack of understanding of precise transcriptional mechanisms regulating the relative timing of waves of CLB activation. In order to address this issue, we employed a simplified mathematical model12 of the cyclin/Cdk1 network to design new experiments addressing how timely waves of CLB expression may occur. Minimal models have been developed, which are able to account for properties of wild type cells.13 With a combined computational and experimental approach we unravelled that a cyclin/Cdk1-dependent regulation of the transcription factor Fkh2 is able to drive timely waves of
mitotic CLB expression. Furthermore, a Boolean model based on the minimal number of interactions needed to capture the information flow of the Clb/Cdk1 network supports the role of an activator molecule in the sequential activation, and oscillatory behavior, of mitotic Clb cyclins. Our data reveal that Clb waves are temporally synchronized by Fkh2-mediated regulation of mitotic CLB genes, and that a Clb/Cdk1-mediated regulation of Fkh2 modulates the CLB cascade.

RESULTS

A transcriptional regulation driving waves of mitotic CLB expression is predicted by kinetic modeling.

To investigate whether a linear cascade of transcriptional activation is compatible with sequential waves of Cib cyclins, we employed a minimal kinetic model previously published by Barberis and colleagues, for which the system property ‘Cib wave formation’, is robust to parameter’s changes. We systematically compared networks that differ in Clb/Cdk1-mediated regulations at mitotic CLB promoters (indicated in red color in Fig. 1a). Specifically, we investigated the role of: (i) Clb5,6/Cdk1 on CLB3,4 transcription (Fig. 1a, arrow A, k_A), (ii) Clb3,4/Cdk1 on CLB1,2 transcription (Fig. 1a, arrow B, k_B), and (iii) Clb5,6/Cdk1 on CLB1,2 transcription (Fig. 1a, arrow C, k_C). Simulation of the minimal network showed alternate Clb waves in time, each wave deriving from the sum of all complexes in which each Clb is present (Fig. 1b; see Supplementary Text for details on model equations and kinetic parameters).

We analyzed three versions of this network, where reactions k_A (Fig. 1c), k_B (Fig. 1d) and k_C (Fig. 1e) were neglected, respectively; conversely, the Clb1,2/Cdk1-mediated feedback loop on CLB1,2 (Fig. 1a, arrow D, k_D) was always present. In Fig. 1c computed time courses of total levels of Clb5,6, Clb3,4 and Clb1,2 are shown when k_A is removed. The simulation revealed no temporal coordination between the times of Cib appearance, with Clb3,4 peaking earlier than Clb5,6. Varying the value of k_A resulted in a non correct order (Supplementary Fig. S1a). We then investigated whether a linear cascade of transcription occurs only through a Clb5/Cdk1-mediated CLB2 promoter. A transcriptional regulation driving waves of mitotic CLB expression is predicted by kinetic modeling.

To investigate the direct role of Fkh1 and Fkh2 in Clb3 regulation, we investigated its protein levels in fkh1Δ or fkh2Δ strains in a time course experiment with G1-elutriated cells (see Supplementary Materials and Methods for details). We compared the minimal network to the versions where only k_A is active (left branch) or where only k_B and k_C are active (right branch). The pairwise comparison of different network structures showed that a change of parameter values affects strongly the distance between peaks of any Clb cyclin (Fig. 1 and Supplementary Fig. S1). If only the left branch is active, time delays tended to be smaller for the distance between Clb5,6–Clb3,4, and Clb3,4–Clb1,2 (Fig. 1f, g) and, consequently, between Clb5,6–Clb1,2 (Supplementary Fig. S1g). Remarkably, for the three peak distances, a positive effect on time delays was observed when only the right branch is active (Fig. 1h, i, Supplementary Fig. S1h), being the correlation indexes close to 1. Together, these analyses predict that regulation of time delays between Clb cyclins, thereby their oscillations, is essentially triggered by a linear cascade of CLB activation.

Fkh2 is responsible for the timely onset of Clb3 protein expression. To investigate the linear cascade of regulation predicted by the kinetic modeling, we tested the role of the transcription factors Fkh1 and Fkh2 that are active in the temporal window where mitotic Clb cyclins are transcribed. Mitotic CLB mRNA levels were measured in fkh1Δ, fkh2Δ, or fkh1Δfkh2Δ strains by quantitative real-time PCR on cells arrested in G2/M phase with nocodazole (Fig. 2a) and in S phase with hydroxyurea (Supplementary Fig. S2b). These treatments revealed reduced CLB1 and CLB2 mRNA levels in fkh2Δ and fkh1Δfkh2Δ mutants and a less prominent effect of fkh1Δ as compared to wild type, as previously observed. This pattern was observed also for CLB3, whereas CLB4 mRNA levels were affected in fkh2Δ and fkh1Δfkh2Δ mutants only in nocodazole treatment but were increased in fkh1Δ cells (Fig. 2a).

Thus, Fkh2 may act as positive regulator of CLB3 and CLB4 transcription. Contrarily, Fkh1 may act as negative regulator of CLB4 transcription, providing further support to earlier experimental and computational analyses proposing that Fkh1 binds to the CLB4 promoter. We then investigated whether Fkh1 and Fkh2 bind to CLB promoters by chromatin immunoprecipitation (ChIP) (see Supplementary Materials and Methods). An enrichment of Fkh2 (Fig. 2b) and Fkh1 (Supplementary Fig. S2c) was observed at CLB1 and CLB2 promoters, as expected. Remarkably, a significant enrichment of Fkh1 and Fkh2 was detected at the CLB3 promoter, consistent with RNA Pol II occupancy data (Supplementary Fig. S2d), but not at the CLB4 promoter. We conclude that Fkh1 and Fkh2 regulate CLB3 transcription. Remarkably, a strong enrichment of Ndd1, coactivator of Fkh2, at the CLB3 promoter was also observed (Fig. 2c), consistent with the fact that Ndd1 is required for Fkh2 periodic activity, and that the Fkh2/Ndd1 complex may regulate CLB3 transcription.

To determine the direct role of Fkh1 and Fkh2 in Clb3 regulation, we investigated its protein levels in fkh1Δ or fkh2Δ strains in a time course experiment with G1-elutriated cells (see Supplementary Materials and Methods for details). In the same yeast strain, the levels of Sic1, Clb5, and Clb2 (the latter not visualized) were used as reference. In the fkh1Δ strain the temporal window of maximal Clb3 expression is similar, but its levels accumulate at a lower amount as compared to wild type (Fig. 2d, e). The fkh2Δ strain instead accumulates Clb3 levels earlier as compared to wild type, and the temporal window of maximal Clb3 expression is delayed, with a protein amount strongly reduced, as compared to wild type (Fig. 2d, e); the delay was also confirmed by FACS analyses (Supplementary Fig. S2e). Notably, Clb3 may be still produced in fkh2Δ cells, indicating that Fkh1 or other, yet unknown transcription factors, may partially overlap with Fkh2 to promote CLB3 transcription. Together, these data confirm our model prediction, and demonstrate the role of Fkh2 in the regulation of the temporal appearance of Clb3 protein levels. Of note, we observe that a premature accumulation of Clb5 protein levels occurs upon Fkh2 deletion, already at early time.
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Fig. 1  Kinetic model of Clb/Cdk1 regulation and computational time courses of total Clb cyclins levels. a Network highlighting in red the sequential transcriptional activation of Clb cyclins, thereby Clb/Cdk1 activities (\(k_a\), \(k_b\), \(k_c\), and \(k_d\)). b Simulations of the network in Fig. 1a are carried out with standard values of parameters, as reported in Supplementary Text for details.\(^{12}\) c–e Model variants were generated starting from the network in Fig. 1a by varying values of \(k_a\) (c), \(k_b\) (d), and \(k_c\) (e), as indicated on each simulation panel and described in the text. The model variants were implemented by ordinary differential equations, with the parameters used for the simulations having the same value among all variants (see Supplementary Text for model description and the full set of equations).\(^{12}\) Simulation panels show the time delay observed between maximum levels (peaks) of Clb cyclins for binary combinations between the minimal model—where Clb/Cdk1 complexes are connected via four transcriptional regulations—and two model variants, independently. f, g Time delay calculated for the left branch (only \(k_c\) active) between Clb5,6 and Clb3,4 (\(t_{3,4}−t_{5,6}\)) (f) and Clb3,4 and Clb1,2 (\(t_{1,2}−t_{3,4}\)) (g). h, i Time delay calculated for the right branch (only \(k_b\) and \(k_a\) active) between Clb5,6 and Clb3,4 (\(t_{3,4}−t_{5,6}\)) (h) and Clb3,4 and Clb1,2 (\(t_{1,2}−t_{3,4}\)) (i). Each parameter of the network may vary from its selected value to the same value multiplied for a random real value comprised between 0.1 and 10, as indicated on each simulation panel.

Fig. 2  Fkh1 and Fkh2 regulate dynamics of mitotic Clb cyclins in a cell cycle-dependent manner. a Quantitative real-time PCR of mitotic CLB transcripts in yeast cells treated with nocodazole (NOC). Total mRNA was isolated from arrested wild type, \(\Delta fkh1\), \(\Delta fkh2\), and \(\Delta fkh1\Delta fkh2\) cells, and CLB1, CLB2, CLB3, and CLB4 mRNA levels were measured. ACT1 and TSA1 genes were used as negative controls, as they are not affected by cell cycle dynamics. Error bars on the histograms represent SDs from the mean of three independent experiments; \(p\)-values are indicated on the histograms in Supplementary Fig. S2a. Nocodazole-arrested cells show that \(fkh2\Delta\) affects both CLB3 and CLB4 transcript accumulation, whereas \(fkh1\Delta\) affects CLB4 transcript accumulation. b, c Binding of Fkh2 (b) and Ndd1 (c) to mitotic CLB promoter regions. Chromatin immunoprecipitation was performed by precipitating protein/DNA complexes from cells grown in exponential phase using an anti-Myc antibody. ACT1 and TSA1 genes were used as negative controls, whereas CLB1 and CLB2 genes as positive controls. Error bars on the histograms represent SDs from the mean of three independent experiments. d, e Fkh2 controls the timing of Clb3 protein expression. d Time course of Clb2-18Mycc, Clb3-TAP, Clb5-HA and Sic1-TAP are shown for wild type (YAN49), \(\Delta fkh1\) (YAG20), and \(\Delta fkh2\) (YAG21) strains. Yeast cells were synchronized by centrifugal elutriation in YPD at 30°C, released into fresh YPD at 30°C, and sampled for western blot analysis at the indicated times. Clb3 levels were quantified in wild type, \(\Delta fkh1\) and \(\Delta fkh2\) strains (e). The experiments were performed at the same time, and the membranes processed identically, with the same antibody aliquots and then exposed to the same levels. \(\Delta fkh1\Delta\) resulted in an decreased amount of Clb3 produced and no time delay as compared to wild type, whereas \(\Delta fkh2\Delta\) resulted in both a decreased amount of Clb3 produced and a time delay as compared to wild type. The result is representative of three independent experiments.
points of the time course (Fig. 2d). However, our quantitative real-time PCR analyses did not show any effect of Fkh2 on CLB5 mRNA levels (see Supplementary Text and Supplementary Fig. S2f). This suggests that other mechanisms, such as a reduced degradation of Clb5 due to a reduced CLB2 transcription/translational and of Clb2/Cdk1 complexes that activate the APC machinery,18 may be responsible for the early accumulation of Clb5 protein levels.

Remarkably, since we discovered that Fkh2 promotes CLB3 transcription, Clb3/Cdk1 may phosphorylate this transcription factor at CLB3 promoter, through a positive feedback loop to produce additional Clb3 protein, and at CLB2 promoter to produce Clb2. In order to explore computationally the possible contribution of these regulations, we investigated their ability to generate the characteristic behavior of the waves of Clb cyclins. Our simulations revealed that a positive feedback loop on CLB3 transcription may provide an additional, but not per se sufficient mechanism to shape Clb waves (see Supplementary Text and Supplementary Fig. S3).

Clb3, but not Clb2, co-localizes with Fkh2/Ndd1 in HU-treated cells. The transcription factor Fkh2 acts in a DNA-bound complex with the transcription factor Mcm1 to regulate cell cycle-dependent expression of the CLB2 cluster, and binding of Fkh2 requires prior binding by Mcm1.3, 10 Since Fkh2 is involved in the transcription of both CLB3 and CLB2 genes, Clb5/Cdk1 may phosphorylate this transcription factor at both promoters. Our kinetic model predicted an involvement of an activator molecule—which we have shown to be the transcription factor Fkh2—by Clb3/Cdk1, in order to promote CLB2 expression. To address this aspect, we first investigated whether Clb3 co-localized with Fkh2. We followed the association of Fkh2 and its coactivator Ndd1 in time by bimolecular fluorescence complementation (BiFC) (see Supplementary Materials and Methods for details and Fig. S4a), after α-factor-mediated synchronization of cells in G1 phase. The presence of a yellow fluorescent signal, also called BiFC signal, highlighted a nuclear localization of the Fkh2/Ndd1 complex formation starting from the early S to G2/M phase (Supplementary Fig. S4b). Specifically, the BiFC signals stably appear at 30 min after α-factor synchronization, which corresponds to the time at which the CLB2 promoter is active following the recruitment of Ndd1 to chromatin in a cell cycle-specific manner.10 Subsequently, Clb3-CFP was integrated in the genome of these cells under control of the endogenous promoter to follow its co-localization with the BiFC signals, and Clb2-CFP was used as control for the experiment. In hydroxyurea-treated cells, Clb3 clearly co-localizes with the BiFC signals, whereas a very low, not localized Clb2-CFP signal is detected (Fig. 3a). The co-localization of Clb2 with the Ndd1/Fkh2 complex occurs later in mitosis, as previously shown (data not shown). This finding indicates that Clb3 co-localizes with the Fkh2/Ndd1 complex before Clb2 accumulation, and suggests a functional interaction between Clb3 and this complex to activate the CLB2 promoter.

Clb3 interacts with Fkh2/Ndd1 and, together with Cdk1, phosphorylates Fkh2. To address the potential association between Clb3 and Fkh2, we performed a Yeast-two-Hybrid assay. Bait (pBTM117) and prey (pACT4) constructs were generated for Fkh2 and Clb cyclins (see Supplementary Materials and Methods for details).12, 20 with the potential interacting partners being overexpressed. Fkh2 full-length and a truncated, C-terminal region of the protein Fkh2\textsubscript{387} (amino acids 387–862) (see Supplementary Fig. S5a for a schematic representation of the constructs used in this study) were tested. Both Fkh2 full-length (Supplementary Fig. S5b) and Fkh2\textsubscript{387} (Fig. 3b) showed a clear interaction with Clb3 as well as with the other Clb cyclins. The interactions between Fkh2\textsubscript{387} and Clb cyclins were validated independently by a GST pull-down assay (Supplementary Fig. S5e).

Subsequently, Fkh2 phosphorylation by the Clb3/Cdk1 complex was tested by immunoprecipitating Clb3/Cdk1 from a yeast lysate that expressed HA-tagged Clb3 and incubating it with bacterially expressed and purified GST-Fkh2 (see Supplementary Materials and Methods for details). We observed that Fkh2 is a substrate of the Clb3-associated kinase activity, as compared to a wild-type strain where Clb3 was not tagged (negative control) (Fig. 3c). Prompted by this result, we have tested whether Clb3/Cdk1 was able to phosphorylate Fkh2 in vivo. To this aim, Fkh2 mobility was detected in a Phos-tag gel in wild type, clb3Δ, clb4Δ, and clb3Δclb4Δ synchronized in G1 phase with a factor. Fkh2 was phosphorylated in a similar manner in all tested strains (Fig. 3d, left panel). Then, we hypothesized that, in absence of Clb3 and Cdk4, Clb2 could replace Clb3 and activate Ndd1 mutation in wild type and clb2Δclb4Δ strains, and observed a clear decrease in Fkh2 mobility in the clb2Δclb3Δclb4Δ strain as compared to the clb2Δ strain, indicating a role for Clb3 in Fkh2 phosphorylation (Fig. 3d, right panel). In order to provide further evidence of the Clb3/Cdk1-mediated phosphorylation on Fkh2, we have tested the kinase activity of Clb3/Cdk1 isolated from a wild-type yeast lysate on a bacterially expressed Fkh2, either full-length or variants carrying single point mutations in two phosphorylation sites (S683 and T697). Both phosphosites are located in the C-terminal region of Fkh2, and have been shown to be phosphorylated by Clb2/Cdk1.10 Deletion of these residues leads to a reduction of the amount of Fkh2 phosphorylated.10 We observed that the Clb3-associated kinase activity is reduced on both mutants between 20% to 40% as compared to a full-length Fkh2 (Fig. 3e), indicating that the phosphosites S683 and T697 mediate the activation of Fkh2 by all Clb/Cdk1 kinase complexes. Altogether, our data confirm that Fkh2 interacts with Clb3, as reported previously,10 and with all Clb cyclins, and show that Clb3/Cdk1 interacts with, and phosphorylates, Fkh2.

Besides interacting with and phosphorylating Fkh2, Clb3/4/Cdk1 may also play a role in Ndd1 phosphorylation in vivo to prime CLB2 transcription. Associations of Ndd1 with Clb2 and Clb3 have been detected in a high-throughput genome-wide screening for complexes,21 but they were not independently validated. To test the Ndd1/Clb2 and Ndd1/Clb3 potential associations, haploid yeast cells expressing the C-terminal region of the Venus protein fused to the C-terminal region of Ndd1 (Ndd1-VC) were transformed with a plasmid carrying the N-terminal region of the Venus protein fused to the C-terminal region of Clb1-4 (VN-Clb1-4). The BiFC signal was observed only for the Ndd1/Clb2 and Ndd1/Clb3 pairs (Fig. 3f). These interactions were further validated by Yeast-two-Hybrid (Fig. S5f) and GST pull-down (Supplementary Fig. S5g) assays, respectively. Thus, we have introduced a scenario showing that Ndd1 is a substrate of the Clb3-associated kinase activity,22 support the hypothesis that the Fkh2/Ndd1 complex may be regulated by all Clb/Cdk1 complexes.

Clb5/Cdk1 and Clb3/Cdk1 are responsible for a sequential mitotic CLB expression

The data presented provide an overall scenario in which the various Clb/Cdk1 complexes are responsible for the transcription of both CLB3 and CLB2 after phosphorylation, and activation, of the transcription factor Fkh2. Thus, this scenario predicts that the first Clb/Cdk1 complex activated, Clb5/Cdk1, would promote the transcription of CLB3 and activation of the next kinase complex in the signaling cascade, Clb3/Cdk1, which ultimately—together with Clb5/Cdk1—would be responsible for the transcription of CLB2 and activation of the last kinase complex of the cascade, Clb2/Cdk1. To investigate the role of each Clb/Cdk1 complex on CLB transcription suggested by both computational and experimental analyses, we tested the influence of Clb5,6 and Clb3,4 on
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**Transmitted light**

- Venus Ndd1/Fkh2
- Cib3-CFP
- Cib2-CFP

**HU treatment**

**α-HA IP + Kinase assay**

- GST-Fkh2
- Clb3-45 KDa
- No tag

**α-GST**

**α-HA**

**IP input (Cdk1)**

**β-Galactosidase**

- SDII
- SDIV

**Release α-factor**

- Wild type
- clb3Δ
- clb2Δ
- clb4Δ

**Histone H1**

- Fkh2_full-length
- Fkh2_S683A
- Fkh2_S697A

**Phosphorylation (a.u.)**

**(+VN-Cib1 / +VN-Cib2 / +VN-Cib3 / +VN-Cib4)**

**Transmitted light**

- Venus Ndd1/Fkh2

**Venus Ndd1/Fkh2**

**Clb3-CFP**

**Clb2-CFP**

**32P**

**α-GST**

**α-HA**

**IP input**

(Cdk1)

**GST-Fkh2**

**Clb3-45 KDa**

**No tag**

**α-GST**

**α-HA**

**IP input (Cdk1)**

**β-Galactosidase**

- SDII
- SDIV

**Release α-factor**

- Wild type
- clb3Δ
- clb2Δ
- clb4Δ

**Histone H1**

- Fkh2_full-length
- Fkh2_S683A
- Fkh2_S697A

**Phosphorylation (a.u.)**

**(+VN-Cib1 / +VN-Cib2 / +VN-Cib3 / +VN-Cib4)**

**Transmitted light**

- Venus Ndd1/Fkh2

**Venus Ndd1/Fkh2**

**Clb3-CFP**

**Clb2-CFP**

**32P**

**α-GST**

**α-HA**

**IP input**

(Cdk1)
both CLB3 and CLB2 transcript levels. Mitotic CLB mRNA levels were measured in fkh2Δ, clb3Δclb4Δ, or clb5Δclb6Δ strains after nocodazole treatment by quantitative real-time PCR. The fkh2Δ strain was used as a control for the experiment, leading to a reduction of both CLB2 and CLB3 mRNA levels as compared to wild type (Fig. 4a), as shown in Fig. 2a. Reduced CLB2 mRNA levels were observed in clb3Δclb4Δ mutants, whereas no CLB3 transcripts were detected, as expected (Fig. 4a). Furthermore, reduced mRNA levels were observed for both CLB2 and CLB3 in clb5Δclb6Δ mutants as compared to wild type, with the former being strongly affected as compared to the latter (Fig. 4a). This result indicates that CLB5,6 promotes CLB3 transcription, and that both CLB5,6 and CLB3,4 impact on CLB2 transcription (Fig. 4a). Thus, this evidence recapitulates both computational and experimental analyses, indicating that both CLB5/Clb1 and Clb3/Clb1 promote CLB2 transcription, and that their progressive activation through Fkh2 guarantees timely waves of Clb cyclins throughout cell cycle progression.

In summary, our data support the hypothesis that the Fkh2/Ndd1 complex may be regulated by all Clb/Cdk1 complexes for the activation of CLB3 and CLB2 promoters (Fig. 4b, colored dotted lines). Regulation mediated by Clb/Cdk1 complexes might occur also on Fkh1/Ndd1 (Fig. 4b, gray dotted lines), as we observed its interaction with some of the Clb cyclins (Supplementary Fig. S6). Remarkably, cooperativity between Ndd1 and Fkh1 was predicted by computational work, and we observed this specific interaction both in vitro and in vivo (Supplementary Fig. S7).

A linear CLB cascade is required to generate temporal oscillations of Clb waves

To theoretically investigate the contribution of the known regulatory interactions of the minimal cyclin network (Fig. 1a) as well as of the newly unraveled transcriptional cascade activating CLB genes to the waves of Clb activation, we employed an independent, qualitative modeling approach, the Boolean modeling, to identify the possible network structure(s) able to reproduce this oscillatory behavior. A prior knowledge network (PKN) of the interactions among four nodes encompassing the mitotic cyclins Clb5, Clb3 and Clb2, and the cyclin-dependent inhibitor Sic1 was modeled (Fig. 5a) following the strategy shown in Fig. 5b for simplicity, each node was assumed to represent the four cell cycle phases: Sic1 (G1), Clb5 (S), Clb3 (G2), and Clb2 (M). This approach, which does not rely on the use of rate constants for the model reactions as compared to kinetic modeling, has been employed to implement all known
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List of all possible interactions

All minimal models

GenYsis

Filter for expected attractors

SQUAD

Filter for expected curves

List of minimal models reproducing experimental observations

MaBoSS

Cross validation

All 6 candidates produce same attractors when simulated with a different qualitative strategy

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MaBoSS

Cross validation

All 6 candidates produce same attractors when simulated with a different qualitative strategy
increasing the Clb2 level. The results are in agreement with the experimental observations.

SQUAD simulations of dosage of Clb2: 1 (Clb3 oscillations when Clb2 is set to 30. It shall be noted that by the computational time significantly reducing cell viability. When the 6 model candidates were tested, two of them (models 2 and 3) were able to reproduce the expected result; the minimal model 2 is shown in Fig. 5c and its simulations are reported in Supplementary Fig. S9d (see Supplementary Fig. S9e for simulations of the minimal model 3). A further increase in Clb2 level reaches a threshold that leads to the inhibition of mitotic exit; this is confirmed by our simulations, showing that in these two models the systems collapses when the level of Clb2 is increased upon a certain level (Fig. 5d). The same behavior was observed for the minimal model 2 (see Supplementary Fig. S9f). The structure of the minimal models 2 and 3 is compatible with a sequential, direct activation of mitotic Clb cyclins (Clb5 → Clb3 → Clb2) to produce periodic Clb oscillations that alternate to Sic1. These findings were also independently validated by applying a probabilistic Monte-Carlo approach (see Supplementary Text for details and Supplementary Fig. S10a–S10c). With this strategy, we verify that the results obtained with SQUAD are not software-specific, but can be extended to different qualitative methodologies.

Together, our analyses support the experimental findings by showing that a linear transcriptional cascade of CLB activation controls the sequential appearance of waves of Clb cyclins.
DISCUSSION

Precise order of cell cycle events is dependent on gradual changes in substrate specificity of cyclin-dependent kinases, which is mediated by phase-specific cyclins.29,31 Sequential activation of cyclins occurs with a characteristic staggered behavior known as waves of cyclins,1, 2 and oscillations in their level ensure a robust regulatory mode can sustain temporal Clb oscillations by creating phosphorylating Fkh2 10 (Fig. 3c, d), and their progressive cascade has been shown to cover a major role in the temporal expression of mitotic cyclin genes (Fig. 6). The coherent feed-forward loop may serve as a delay element: it responds rapidly to stimuli in one direction (from Clb2 ON to Clb5 OFF for cell cycle re-entry), and at a delay to steps in the opposite direction (from Clb2 OFF to Clb5 ON for cell cycle progression). This may allow for a rapid response and sustained oscillations.38 Feed-forward loops may be seen as a multistep ultrasensitivity,39 where small changes in the level or activity of X can be amplified at the target gene Z because of the combined action of X and Y. Of note, within this regulatory motif, a Clb3/Cdk1-mediated positive feedback loop on CLB3 transcription (Y→Z) may contribute to timely shape certain Clb waves, without being sufficient per se to generate their oscillatory pattern, for which presence of the direct regulation of Clb3/Cdk1 on CLB2 transcription (Y→Z) is required. However, it is at present not known whether CLB2 expression is dependent on the accumulation of adequate levels or activities of both Clb5/Cdk1 and Clb3/Cdk1 in a threshold-like fashion.

In conclusion, our findings suggest a potential involvement of differential phosphorylation mechanisms40 mediated by various Clb/Cdk1 activities for the Fkh1/Fkh2-dependent regulation of phase-specific CLB genes. These details have been not yet elucidated, and are currently under investigation in our laboratory. Remarkably, although the C-terminal domain of Fkh2 (amino acids 458–862)—which is missing in Fkh1—contains the majority of Clb/Cdk1 target sites,10 it does interact weakly with Clb cyclins (Supplementary Fig. 5Sb). Contrarily, a longer fragment (amino acids 387–862) including a part of the forkhead DNA-binding domain (FKH) revealed a strong interaction with all Clb cyclins (Fig. 3b), thus suggesting that recruitment of Fkh2 is potentially mediated by this region. We speculate that a cooperativity of Clb/Cdk1-dependent phosphorylations could promote the activation of Fkh2 in order to drive timely waves of CLB expression. This regulatory mode coupling Clb/Cdk1 activity and transcription may fine tunes the precise cell cycle timing, pinpointing a design principle in cell division.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains, plasmids and growth conditions used in this study are described in Supplementary Materials and Methods.

Cell synchronization, cytometry analysis, and western blot

Cell synchronization, cytometry analysis, and western blot were performed as previously described19, 26 with modifications as described in Supplementary Materials and Methods.

In vitro kinase assays

For the assay in Fig. 3c, GST-Fkh1 and GST-Fkh2 were expressed in E. coli and purified using glutathione-Sepharose beads. To obtain the Clb3/Cdk1 complex, the W303 strain and a strain expressing Clb3-HA from its chromosomal locus were used. For the assay in Fig. 3e, Clb2/Cdk1 and Clb3/Cdk1 complexes were isolated from the W303 strain. Yeast cells were transformed with plasmids expressing C-terminal TAP-tagged Clb2 and Clb3 under galactose (GAL1) promoter (pRSAB1234GAL-CLB2-TAP and pRSAB1234GAL-CLB3-TAP). The kinase assays were performed as described in Supplementary Materials and Methods.

In vivo phosphorylation assay

For the assay in Fig. 3d, yeast cells were grown at 25 °C in yeast extract peptone dextrose (YPD) and synchronized in G1 phase with α-factor. Samples were collected every 10 min for 90 min after synchronous release, and extracted proteins of the most relevant time points were applied to a 6% polyacrylamide gel electrophoresis (PAGE) gel added with Phos-tag.
GenYsis, which uses ef Materials and Methods. Boolean simulations were performed with MaBoSS, which is based on continuous time Markov processes applied decision diagram algorithm to identify all the steady states of the system. Previous work was supported by the SLS Starting Grant of the University of Amsterdam, UvA to M.B., by the UvA-Systems Biology Research Priority Area grant to M.B., and by the ENFIN Network of Excellence grant funded by the European Commission to M.B. (within contract number LSHG-CT-2005-518254 to E. Nasmyth, K. Control of the yeast cell cycle by the Cdc28 protein kinase. Cell Biol. Cell. Biol. 10, 986–988 (2000).

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