Periodic Cyclin-Cdk Activity Entrains an Autonomous Cdc14 Release Oscillator

Ying Lu[*] and **Frederick R. Cross**

The Rockefeller University, 1230 York Avenue, New York, New York 10065, USA

SUMMARY

One oscillation of Cyclin-dependent kinase (Cdk) activity, largely driven by periodic synthesis and destruction of cyclins, is tightly coupled to a single complete eukaryotic cell division cycle. Tight linkage of different steps in diverse cell cycle processes to Cdk activity has been proposed to explain this coupling. Here we demonstrate an intrinsically oscillatory module controlling nucleolar release and re-sequestration of the Cdc14 phosphatase, which is essential for mitotic exit in budding yeast. We find that this Cdc14 release oscillator functions at constant and physiological cyclin-Cdk levels, and is therefore independent of the Cdk oscillator. However, the frequency of the release oscillator is regulated by cyclin-Cdk activity. This observation together with its mechanism suggests that the intrinsically autonomous Cdc14 release cycles are locked at once-per-cell-cycle through entrainment by the Cdk oscillator in wild type cells. This concept may have broad implications for the structure and evolution of eukaryotic cell cycle control.

INTRODUCTION

Periodicity of eukaryotic cell cycle events is driven by oscillations of cyclin-dependent kinase (Cdk) activity (Morgan, 2007). Checkpoint surveillance mechanisms enforce correct ordering of events by delaying subsequent events until previous ones are finished; however, checkpoints are not generally essential for ordering the unperturbed cell cycle (Elledge, 1996; Weinert et al., 1994).

Cdk activity oscillation can order cell cycle events by a 'ratchet'-like mechanism: high cyclin-Cdk activity triggers the initiation of a cell cycle event, but inhibits its completion or re-initiation (Nasmyth, 1996; Stern and Nurse, 1996). Therefore, as a result of having different thresholds for cyclin-Cdk, cell cycle events occur in order, exactly once per cyclin-Cdk cycle. This mechanism is well established in control of DNA replication (Kearsey and Cotterill, 2003), and similar ratchet-like mechanisms may apply to processes such as spindle and bud morphogenesis (Bloom and Cross, 2007).

Corresponding author: F. Cross, 1-212-327-7685, fcross@rockefeller.edu.
[*]Present address: Harvard Medical School, Department of Systems Biology, Boston, 02115, USA

Independent of molecular mechanisms, ratchet control entails that locking Cdk activity at any constant level should arrest the controlled process at a specific step. However, some cell cycle events may occur cyclically without oscillation of mitotic cyclin-Cdk activity, such as SPB/centrosome duplication cycles, and periodic budding and cell-cycle-regulated transcription in budding yeast (Gard et al., 1990; Haase and Reed, 1999; Haase et al., 2001; McCleland and O'Farrell, 2008; Sluder et al., 1990). Such 'endocycles' pose a challenge to the concept of cyclin-Cdk-based ratchet control, but the relevance of these endocycles to the mitotic cell cycle, and what mechanism, if any, entrains them to mitotic cyclin-Cdk cycles, remains unclear

Cdc14 is an essential mitotic phosphatase in budding yeast. Cdc14 is restrained and inhibited in the nucleolus by the constitutively nucleolar Net1p, except in mitosis (Shou et al., 1999; Visintin et al., 1999). The spindle orientation checkpoint (SPOC, regulating the mitotic exit network MEN) and cyclin-Cdk oscillation can regulate Cdc14 localization and activity (Azzam et al., 2004; Bardin et al., 2000; Jaspersen and Morgan, 2000; Pereira et al., 2000; Queralt et al., 2006; Stegmeier and Amon, 2004; Stegmeier et al., 2002). Disrupting SPOC control by removing the Bub2 inhibitor has almost no effect on Cdc14 release in unperturbed cell cycles, suggesting that mitotic cyclin (Clb)-Cdk oscillations may play an important role in regulating Cdc14 release timing. However, it is as yet unclear how Cdc14 localization responses to different Clb levels, and whether these controls constitute a ratchet mechanism sufficient to lock Cdc14 release to once per cell cycle.

To understand whether Clb-Cdk oscillations control Cdc14 localization through a 'ratchet'-like mechanism, we locked mitotic cyclin Clb2 at stable physiological levels, by titrated pulses of undegradable Clb2, and correlated Cdc14 release and mitotic exit (ME) events to Clb2 levels in individual cells, following a recently developed procedure (Drapkin et al., 2009). High Clb2 blocks ME (Surana et al., 1993); however, the peak level of Clb2-Cdk activity attained in a normal cell cycle was inefficient at restraining ME (Drapkin et al., 2009), posing a problem for the simple cyclin-based ratchet model. Sharpening this contrast, here we show that Cdc14 cycles in and out of the nucleolus multiple times at high but physiological fixed mitotic cyclin levels. These and other results lead us to propose that Cdc14 release, and likely other cell cycle processes, are controlled by intrinsically oscillatory modules, that are entrained to a single occurrence at appropriate cell cycle positions by cyclin-Cdk cycles through a 'phase-locking' mechanism.

RESULTS

Blocking mitotic exit with undegradable Clb2kd reveals Cdc14 release endocycles

We determined the response of the Cdc14 release cycle to fixed cyclin-Cdk levels (Drapkin et al., 2009), using a quantitative, single cell measurement for Cdc14 localization based on variation of cellular Cdc14-YFP pixel intensities, standardized to variation of nucleolar Net1-mCherry (Lu and Cross, 2009) (Experimental Procedures; Fig. 1A).

We blocked cells in metaphase by depleting the essential anaphase-promoting complex (APC) activator Cdc20, by shutoff of *MET3-CDC20* (Sullivan et al., 2001). Cdc20 promotes proteolysis of the anaphase inhibitor Pds1, and initial proteolysis of B-type cyclins

(Shirayama et al., 1998; Wäsch and Cross, 2002; Yeong et al., 2000). We briefly pulsed *cdc20*-blocked cells with stable mitotic cyclin Clb2kd-GFP lacking destruction and KEN boxes recognized by the APC activators Cdc20 and Cdh1 (Pfleger and Kirschner, 2000; Schwab et al., 1997; Visintin et al., 1998; Wäsch and Cross, 2002). Stable Clb2kd-GFP accumulates in these cells to a level averaging around the peak Clb2-GFP level in a wild-type cell cycle ('1X peak') (Fig. S1A).

Re-inducing *MET3-CDC20* induced anaphase, which proceeded on schedule independent of stable Clb2kd-GFP (data not shown). Clb2kd-GFP and associated kinase activity was constant through this protocol (Drapkin et al., 2009). We assayed post-anaphase events as a function of single-cell Clb2kd-GFP levels (Fig. 1C).

>=1X peak Clb2kd-GFP induced dose-dependent delays in cytokinesis and bud emergence (Fig. S1B)(Drapkin et al., 2009). In contrast, Cdc14-YFP was released from the nucleolus and subsequently resequestered, with essentially normal kinetics, up to at least 3X peak Clb2kd-GFP (Fig. 1D) (Drapkin et al., 2009). Strong overexpression of stable Clb2 was shown previously to cause extended Cdc14 release (Stegmeier et al., 2002). We confirmed this observation with continuous galactose induction of *GAL1-CLB2kd* (yielding >=10-fold peak Clb2kd levels (Fig. S1C)). However, in the following we pursue only results obtained at approximately physiological Clb2kd levels.

Remarkably, cells containing near-1X peak Clb2kd-GFP frequently exhibited multiple 'endocycles' of Cdc14-YFP release and resequestration, before finally undergoing cytokinesis and rebudding (Fig. 1B,D; Supplemental Movie 1).

Cdc14 endocycles occurred without budding, cytokinesis, or nuclear or nucleolar division. The G1 cyclin *CLN2* is expressed at cell cycle Start (Wittenberg et al., 1990). We observed no significant *CLN2pr-GFP* expression (Bean et al., 2006; Mateus and Avery, 2000) in cells undergoing Cdc14 endocycles, while a burst of *CLN2pr* expression occurred when these cells finally budded (Fig. 1E).

In yeast, cyclin-Cdk phosphorylation excludes the MCM helicase complex from the nucleus except in M/G1, helping to restrict pre-replicative complex formation to pre-S phase (Kearsey and Cotterill, 2003). ~1X peak Clb2kd-YFP was sufficient to completely block Mcm2-GFP nuclear re-accumulation (Drapkin et al., 2009), confirming continuous high Clb2kd-associated kinase in this protocol. Consistent with this observation, we observed little or no DNA endoreduplication during Cdc14 endocycles by DNA flow cytometry (Fig. S1D).

Endogenous Clb2-GFP was uniformly degraded after initial Cdc14 release, regardless of exogenously pulsed Clb2kd levels. Endogenous Clb2-GFP remained low during Cdc14 endocycles, only reaccumulating upon cessation of endocycles (Fig. S1E).

Cdc14 endocycles are not driven by oscillations of Sic1, the stoichiometric Clb-Cdk inhibitor (Mendenhall and Hodge, 1998), because Sic1 levels in Clb2kd-pulsed cells were insufficient for Clb2kd inhibition (Drapkin et al., 2009). Consistently, Cdc14 endocycles

were independent of Swi5, the major *SIC1* transcription factor (Toyn et al., 1997) (Fig. S3A).

Thus, Cdc14 release endocycles are not due to oscillations of Clb2kd-Cdk or of endogenous G1 or mitotic cyclins. Cdc14 endocycles occur in the absence of cytokinesis, rebudding, Mcm complex nuclear reaccumulation and DNA replication.

Frequency control of the Cdc14 release endocycle by Clb2kd levels

Clb2kd-GFP at less than ~0.5X peak had little effect on cell-cycle progression (Drapkin et al., 2009), and a single normal cycle of Cdc14 release and resequestration followed anaphase (marked by nucleolar Net1 separation) (Fig. 2A, category 1). Cdc14 release in the next cell cycle was frequently delayed, possibly due to 2nd-cycle defects caused by low Clb2kd (Drapkin et al., 2009). At ~0.75X peak levels of Clb2kd-GFP, ME was delayed but not blocked (Drapkin et al., 2009), and a 2nd Cdc14-release event sometimes occurred rapidly after bud emergence, without associated anaphase (Figs. 1D;2A; category 2). At >=1X peak Clb2kd concentrations, we observed Cdc14 endocycles: repeated Cdc14 release and sequestration without evident cell cycle progression (Fig. 2A, category 3).

Category 2 cells are intermediate between normal tight linkage of Cdc14 release to cell cycle progression, and complete uncoupling as in category 3 endocycling cells, suggesting a continuous transition from normal Cdc14 release cycles to endocycles with increasing Clb2kd.

Below ~$1.7\times$ peak Clb2kd, Cdc14 release frequency correlated positively with Clb2kd-GFP level (Fig. 2B; $P<2*10^{-7}$) using only data from 'category 3' cells that undergo endocycles without apparent cell cycle progression. A quantitatively similar, highly significant positive correlation was observed using all three categories of cells, suggesting that Clb2 levels may directly control Cdc14 release frequency, independent of cell cycle progression. At Clb2kd $>1.7\times$ peak, endocycle frequency saturated at once per ~45 min. Despite considerable noise in the data, we estimated the slope of the Clb2kd/endocycle frequency relationship (Fig. 2B legend) with a linear function ($k=0.013\pm0.004/\text{Peak}_{Clb2}{}^{-1}\text{min}^{-1}$).

Although Clb2kd levels controlled Cdc14 release frequency, the amplitude and duration of release was independent of Clb2kd (Fig. S2A). Those observations are important for the theoretical analysis below.

Requirements for Cdc14 endocycles

A B-type cyclin-independent oscillator may drive G1 events (Haase and Reed, 1999; Orlando et al., 2008). To determine if this oscillator might also drive Cdc14 endocycles, we overexpressed the stable Clb-Cdk inhibitor Sic1-4A to inhibit all B-type cyclin-Cdk activity (Verma et al., 1997). Cdc14 remained nucleolar, despite multiple budding without cell division (manifesting the G1 oscillator (Haase and Reed, 1999)) (Fig. S3E). Additionally, we constructed a *cdc14-1* temperature-sensitive strain with all B-cyclins deleted. We collected small G1 cells by elutriation, and incubated them at non-permissive temperature for *cdc14-1*. *cdc14-1* and *CDC14* B-cyclin-depleted cells budded and rebudded with

identical kinetics (Fig. S3F). Therefore, the Cdc14 release endocycle and the G1 endocycle are independent.

Polo kinase Cdc5 is required for Cdc14 release (Visintin et al., 2003), and Cdc14 endocycles required active Cdc5, even after an initial round of Cdc14 release (Fig. 3D). The FEAR network and MEN are required for early and late anaphase Cdc14 release respectively (Azzam et al., 2004; Queralt et al., 2006; Stegmeier and Amon, 2004). The MEN component Cdc15 was required for Cdc14 endocycles, but neither the FEAR network component Spo12, nor the key FEAR pathway event of Net1 phosphorylation by Clb-Cdk, was required (Fig. S3B,C). Thus, Cdc14 endocycles in Clb2kd-blocked cells may involve mechanisms controlling late-anaphase Cdc14 release in the free-running cell cycle, since both require Cdc5 and Cdc15, but not FEAR pathway components. In *bub2* cells, MEN activity is likely near-constitutive (Alexandru et al., 1999; Pereira et al., 2000; Xu et al., 2000). Cdc14 release endocycles persisted in Clb2kd-arrested *bub2* cells (Fig. S3D), suggesting that these endocycles do not require cyclical inactivation of the SPOC.

Cdc5 is down-regulated at the end of mitosis by transcriptional inactivation and by Cdh1-APC-dependent proteolysis (Charles et al., 1998; Shirayama et al., 1998), and Cdc5 proteolysis promotes Cdc14 resequestration (Visintin et al., 2008). To determine whether Cdc14 resequestration in endocycles similarly required Cdc5 proteolysis, we tested the Cdh1-resistant Cdc5-*NT* mutant (Visintin et al., 2008). In *CDC5-NT* cells deduced to contain >1X peak Clb2kd, we did not detect multiple Cdc14 release endocycles; 22% of cells showed extended Cdc14 release, and 53% of cells had a short Cdc14 release/re-sequestration cycle prior to a long release (Fig. 3A). These very long release periods in *CDC5-NT* cells contrasted with the normal duration of Cdc14 release in endocycling *CDC5+* cells (see above).

Cdh1 is required for Cdc5 degradation, and *cdh1* but not *CDH1* cells containing 0.5X to 2X peak Clb2kd released Cdc14 for up to 80 minutes, and did not exhibit Cdc14 endocycles (Fig. 3B). Surprisingly, however, low-amplitude Cdc14 endocycles occurred in *cdh1* cells with > 2X peak Clb2kd (Fig. 3B). This result suggests a parallel Cdh1-independent pathway allowing Cdc14 endocycles (see Discussion); but the primary mechanism likely requires Cdh1-dependent proteolysis of Cdc5, dependent on the Cdc5 N-terminal destruction box.

Cdc14-Cdh1-Cdc5 negative feedback may contribute to Cdc14 endocycles

Cyclical activation and inactivation of Cdc5 may be obligatory for Cdc14 endocycles, since endocycles are prevented by either constitutively high (*CDC5-NT*) or constitutively low Cdc5 (*cdc5-1*).

We hypothesized a negative feedback loop to explain Cdc14 endocycles: Cdc5 promoting Cdc14 release, Cdc14 activating Cdh1 leading to Cdc5 proteolysis, and consequent Cdc14 resequestration. In the wild-type cell cycle, Cdc5 cannot reaccumulate until the next M-phase: *CDC5* is co-transcribed with *CLB2* under Clb2-dependent positive feedback control (Wittenberg and Reed, 2005), so Clb2 removal at anaphase blocks further *CDC5* transcription. In Clb2kd-blocked cells, persistent Clb2kd could both maintain *CDC5* transcription, and inactivate Cdh1 by phosphorylation (in the absence of counterbalancing

dephosphorylation by Cdc14), allowing rapid reaccumulation of Cdc5 and endocyclic Cdc14 release and resequestration. This negative feedback hypothesis could explain absence of endocycles in *cdc5-1*, *CDC5- NT* and *cdh1* cells.

We used a cell-cycle-regulated *CDC5pr-GFP* reporter (J. Skotheim, pers. comm.) to measure *CDC5* transcription. Consistent with the negative feedback hypothesis, we observed higher trough levels and rapid rebound of *CDC5* transcription oscillation in Clb2kd-pulsed cells compared to unpulsed controls (Fig. 3C). Also consistent with the hypothesis, Cdc14 endocycles required new protein synthesis, since endocycles were blocked by addition of cycloheximide after an initial Cdc14 release (Fig. 3D).

Finally, in cells undergoing Cdc14 endocycles, we observed cyclical accumulation and degradation of Cdc5-GFP fusion protein expressed from the endogenous promoter, out of phase with Cdc14 release (Fig. 3E; the actual peak of Cdc5-GFP protein is likely to be ~15 min. earlier than the observed peak due to the time required for fluorescent maturation of newly synthesized Cdc5-GFP).

A qualitative ODE model (Fig. 3F;S3G) of a Cdc5-Cdc14-Cdh1 negative feedback loop reproduced our major results, including dependence of Cdc14 endocycle frequency on Clb2kd concentrations (Fig. 2B).

The experiments above suggest that Cdc14 localization could be controlled by an oscillatory module. Cdc14 release endocycles could come about because Clb2kd accelerates the Cdc14-release-control module, by driving rapid rebound of the Cdc5-Cdc14-Cdh1 negative feedback oscillator (Fig. 2B;4A), and blocks Clb-Cdk oscillation, resulting in a temporal separation of the two oscillators.

Elucidating the mechanism of Cdc14 endocycles does not address whether the potential for Cdc14 endocycles is physiologically relevant in the normal cell cycle. To address this issue required additional experimental and analytical studies.

Oscillator uncoupling reveals Cdc14 endocycles in free-running cell cycles

Does the oscillation of this Cdc14 module control timing and execution of *normal* Cdc14 release in unperturbed cells cycles? Any manipulation trying to study the autonomous behavior of Cdc14-control module will inevitably perturb cell cycle progression. So, we analyzed this question in two steps: first, we asked whether the Cdc14-control module could oscillate autonomously in free-running cell cycles; second, we studied whether oscillatory behavior of the intact module might be involved in controlling normal Cdc14 release events.

First, we tried to uncouple the Cdc14 module from the Clb-Cdk oscillator, without blocking the cell cycle. Genetic manipulations providing sufficient uncoupling could allow detection of Cdc14 release endocycles in free-running cell cycles. Plausible coupling components include: 1. *CDC5* transcription, activated by Clb-Cdk; 2. Cdh1, activated by Cdc14 and degrading Clbs and Cdc5; 3. the MEN, indirectly activated by mitotic cyclin-Cdk, since anaphase (promoted by cyclin-Cdk (Fitch et al., 1992)) activates the MEN (Fig. 4A).

We made *CDC5* transcription independent of Clb-Cdk, using *GAL1-URL-CDC5* ((Visintin et al., 2008); the destabilizing *URL* tag kept Cdc5 at a non-lethal level). Consistent with the oscillatory module hypothesis, in freely cycling *GAL1-URL-CDC5* cells, we observed G1-specific Cdc14 endocycles in ~70% daughter cells (Fig. 4B). G1 cells stably contain almost no cyclin-Cdk activity (Mendenhall and Hodge, 1998), so events directly controlled by a cyclin-Cdk ratchet have no basis for repeated activity in G1 cells.

We did not observe Cdc14 endocycles or Cdc14 release anomalies in cycling *cdh1* cells (data not shown), perhaps because Cdh1 contributes to the Cdc14 intrinsic oscillatory mechanism, as well as to its coupling with Cdk oscillations (Fig. 3B). More complete decoupling could reveal Cdc14 endocycles in the absence of Cdh1, assuming parallel coupled pathways within the Cdc14 oscillatory module (see above). Indeed, additionally deletion of *BUB2*, eliminating dependence of MEN activation on mitotic cyclin-induced anaphase (Fesquet et al., 1999; Fitch et al., 1992; Pereira et al., 2000), revealed G1-specific Cdc14 endocycles in cycling *cdh1 bub2* cells (Fig. 4B). *BUB2* deletion had little or no effect on its own or in *GAL1-URL-CDC5* cells (Fig. S4A,B). *CDC5- NT* in a *CDH1 BUB2* background also induced G1 Cdc14 endocycles (Fig. S4C).

Daughter preference of endocycles could be due to G1-cyclin-dependent relocalization of MEN activators such as Lte1 to the incipient bud (Jensen et al., 2002), sequestering these activators until spindle pole entry into the bud in the next anaphase. Mother cells bud and express G1 cyclins much earlier than daughters (Di Talia et al., 2007), which could forestall mother G1 Cdc14 endocycles. Actin depolymerization with latrunculin B (LAT-B) prevents Lte1 sequestration by inhibiting budding; LAT-B treatment also inhibits all overt cell cycle progression (Jensen et al., 2002). *cdh1 bub2* cells incubated with LAT-B exhibited Cdc14 endocycles in unbudded mothers and daughters with equal probability (Fig. 4C) (up to four endocycles, usually without associated anaphase), supporting the idea that rapid bud emergence and sequestration of MEN activators could prevent Cdc14 endocycles in G1 mother cells.

Therefore we conclude that the Cdc14-release module can oscillate autonomously in free-running cell cycles. These observations also emphasize the importance of oscillator coupling for maintaining timely, once-per-cell-cycle Cdc14 release in the normal cell cycle.

The Cdc14-release module is intrinsically oscillatory at mid-cell-cycle Clb-Cdk levels

The minimal Clb2 level allowing oscillation of the Cdc14 module determines whether this oscillatory behavior could control normal Cdc14 release in unperturbed cell cycles. If this level is low, oscillation of the module will last for most of the cell cycle, implying a likely role in controlling normal Cdc14 release timing and execution.

To determine this critical level, we used LAT-B to inhibit cytokinesis, budding and anaphase, to study the autonomous behavior of the Cdc14 module at low Clb2kd levels. We also deleted *BUB2* to avoid the interference of SPOC. *BUB2* deletion alone caused no difference in Cdc14 endocycles (Fig. S4A). In LAT-B-treated *bub2* cells, 90-min period Cdc14 release cycles emerged at 0.2X peak Clb2kd-GFP (Fig. 4D). With <1X peak Clb2kd, 39/46 cells exhibited Cdc14 endocycles. The frequency-Clb2kd relationship had a similar

slope as was obtained in Fig. 2B at higher Clb2kd levels (Fig. S5). Therefore, the Cdc14-release module oscillates even at Clb2 levels characteristic of early-mid cell cycle, with a frequency very close to normal Cdc14 release cycles (~90 min.), consistent with the oscillator functioning to control Cdc14 release timing in normal cell cycles. The apparently continuous transition from normal Cdc14 release cycles to endocycles also support this conclusion (Fig. 2A).

Control of Cdc14 release by an autonomous oscillator must be compatible with the well-established restriction of Cdc14 release to late mitosis, at the end of the cyclin-Cdk cycle. We show that coupling of the autonomous oscillation of the Cdc14 module to Clb-Cdk is sufficient to yield appropriate regulation and timing of Cdc14 release in the unperturbed cell cycle; this, coupled with the likelihood that the Cdc14 oscillator is functioning during much of the normal cell cycle (see above), makes the Cdc14 oscillator an important candidate for regulating Cdc14 release timing in the normal cell cycle. We extend this concept to oscillatory modules controlling other cell cycle events.

Cyclin-Cdk oscillations could order cell cycle events by phase-locking

Other cell cycle events besides Cdc14 release can occur 'endocyclically', driven by intrinsic oscillating modules without mitotic cyclin-Cdk oscillation. These observations are difficult to fit into Cdk-ratchet models (see Introduction). We considered alternative, non-ratchet-like ways that cyclin-Cdk oscillations could order cell cycle events.

Physiological Clb2kd concentrations linearly controlled Cdc14 release endocycle frequency (Fig. 2B). Oscillators with different *intrinsic* frequencies can be synchronized to oscillate at one collective frequency if the frequency of one oscillator is sufficiently controlled by the activity of another, a phenomenon called 'phase-locking (PL)' (Glass, 2001; Winfree, 1967). A well-known biological example of PL is the entrainment of the circadian clock to diurnal cycles. Cyclin-Cdk oscillations could similarly entrain or phase-lock Cdc14 release, as well as other autonomous oscillatory modules, promoting orderly and coherent cell cycle progression (Fig. 5A).

To demonstrate how PL could order cell cycle events, we built a conceptual model containing four oscillating modules: the cyclin-Cdk oscillator, and three autonomous 'peripheral' oscillators controlling specific cell cycle events (e.g., budding, SPB duplication, Cdc14 release). Without coupling, different intrinsic frequencies of the peripheral oscillators results in a disordered and irregular sequence of cell cycle events (Fig. 5B, left). In simulation, allowing cyclin-Cdk master oscillator to affect the frequencies of the peripheral ones resulted in all oscillators cycling exactly once per cyclin-Cdk cycle, in a fixed order (Fig. 5B, right).

This result of a distinct, dynamically *stable* PL solution for each oscillating module, provided they are coupled to the central Cdk oscillator, is derived from a very simple model, but may be robust to biological complexity in real systems. This is because PL is largely independent of detailed mechanism. Stability is determined by the local dynamical structure near the fixed point (Winfree, 1967), given sufficiently strong linkage, and reasonably close intrinsic frequencies. These features may be highly evolvable (see Discussion).

Reducing amplitude of the cyclin-Cdk oscillator results in disordered cell cycle events

PL models (Fig. 5B) lead to a key prediction: lowering the amplitude of Clb-Cdk oscillation should weaken entrainment between the Clb cycle and other oscillators and perturb *order* of PL-controlled cell-cycle events (Fig. 6A). Cell cycle control through ratchet-like mechanisms yields entirely different predictions: lowering amplitude should block or delay events, but should not alter event order. In both cases these considerations are largely independent of model details.

We tried two experimental strategies to lower the amplitude of cyclin-Cdk oscillation. We first used strong constitutive overexpression of the Sic1 inhibitor of Clb-Cdk activity (Mendenhall and Hodge, 1998). The *SIC1* overexpression cassette we used ('*2X GAL-SIC1*') was capable of significantly reducing Clb-Cdk levels in cells previously blocked with approximately peak Clb-Cdk activity, consistent with the idea that this cassette will similarly lower the peak in cycling cells (Fig. S6A; Fig. 6B). *2X GAL-SIC1* cells are fully viable. As a contrasting means to lower amplitude, we deleted *CDH1* and *SIC1*, which results in a high trough level of Clb2-Cdk activity but probably little reduction in the peak (Wäsch and Cross, 2002; Yeong et al., 2000) (Fig. 6B). Although *cdh1 sic1* cells are ultimately inviable (Schwab et al., 1997; Visintin et al., 1997), they undergo multiple cell cycles (Wäsch and Cross, 2002), allowing analysis of order of events by time-lapse microscopy. Thus, in *GAL1-SIC1* cells, Clb-Cdk levels are extremely low during an extended G1, then probably rise to a reduced peak compared to wild-type, while in *cdh1 sic1* cells there is probably no period with low Clb-Cdk levels, but likely a relatively normal peak level.

These two mechanisms for reducing the amplitude do so by opposite means: lowering the peak, vs. raising the trough. Ratchet control, in which specific Clb-Cdk levels control sequential steps in a process, predicts very different results from these manipulations, since a ratchet mechanism demands distinct responses to different Clb-Cdk activity levels. In contrast, the PL perspective can predict qualitatively similar outcomes of event disorder in these two cases.

To focus specifically on reduction of amplitude of the Clb-Cdk oscillation, we deleted the spindle orientation checkpoint component *BUB2*, to relieve dependence of Cdc14 release on spindle position (Bardin et al., 2000) (Qualitatively similar although lesser effects on budding, cytokinesis, and anaphase were observed in a *BUB2* background; *bub2* deletion alone had no effect (Fig. S4A)).

GAL1-SIC1 expression in *bub2* cells resulted in 64% abnormal first cell cycles after Sic1 expression on galactose medium (supernumerary Cdc14 release without nucleolar separation; extra budding without Cdc14 release or cell division; or cytokinesis without Cdc14 release or nucleolar separation (Fig. 6B)). Remarkably, similar cell cycle anomalies with close frequencies were also observed in cycling *cdh1 sic1 bub2* cells. Those anomalies are not likely due to nonspecific growth defects since *GAL1-SIC1 bub2* cells grow well both in bulk culture and when monitored as single cells. *cdh1 sic1* occasionally causes pre-anaphase block, probably due to replication problems (Wäsch and Cross, 2002); such cells were excluded from analysis.

The conceptual PL model yielded loss-of-order phenotypes as a result of lowering the amplitude of Cdk oscillation (Fig. 6A). In contrast, any Cdk-ratchet model would predict that under amplitude changes, the normal orders of cell cycle events would remain the same, excepting delays or blockages of some events. Further, near-opposite phenotypes would be predicted for lowering the peak vs. raising the trough. Thus, the result of out-of-order phenotypes, and the surprising similarity of the phenotypes of two entirely distinct ways of reducing amplitude, support a PL model over a ratchet model.

The PL model describes how Cdk oscillation entrains autonomous peripheral oscillators to form a coherent cell cycle, associating once-per-cell-cycle occurrence of an event (e.g. Cdc14 release, SPB duplication) in normal cell cycles with its capacity to undergo endocycles in blocked cells In contrast, in any ratchet model, endocycles must be pathological consequences of cell cycle blockage. Therefore, an empirical test of the PL vs. the ratchet perspectives is to ask whether the response of the oscillatory module to fixed Clb-Cdk levels is consistent with timing in the normal cell cycle. We calculated the stable phase offset (the difference between peak Clb2 and peak Cdc14 release), based on a simple quantitative PL model, containing only three parameters extracted directly from empirical observations on Clb2kd-blocked cells (Fig. 2B; Supplemental Methods). (For example, one key parameter, strength of coupling of Clb2 to Cdc14 cycle frequency, is measured from the slope of the straight-line fit in Fig. 2B.) An analytical solution yielded a phase-offset with Cdc14 release occurring as Clb-Cdk levels decreased (Supplemental Methods; Fig. 6C), consistent with experiment (Stegmeier and Amon, 2004). Uncertainties in parameter estimation do not significantly affect this conclusion, since increasing or decreasing the parameter estimates by one standard deviation of measurement error, alone or in combination, had little effect (Fig. 6C). This fit would be unlikely if endocycles are pathological consequences of cell cycle blockage.

Incorporating the inhibitory effects of Cdc14 release on Clb-Cdk activity is likely to further stabilize the PL between Cdc14 and Cdk oscillators. We lack sufficient information to determine all parameters in the 'bidirectional' model, but the same general conclusion is reached (Supplemental Methods).

Any oscillating module whose frequency is controlled by Cdk can potentially be phase-locked. We constructed a similar analytical model for PL of a hypothesized SPB duplication module. Published data are insufficient to obtain empirical estimates of parameters. Still, we could estimate predicted timing using our model and plausible parameter choices (Supplemental Methods). SPB duplication was predicted to occur early in the cell cycle, before the rise in Clb-Cdk, as observed experimentally (Fig. 6C), showing consistency of timing of SPB duplication with a PL model.

DISCUSSION

Cdc14 endocycles, oscillatory modules, and phase-locking

The canonical ratchet model for once-per-cell-cycle regulation by cyclin-Cdk oscillations proposes that high and low cyclin-Cdk levels promote alternating steps of a process. In this case, any fixed level of cyclin-Cdk should result in a terminal arrest with one or the other

steps of each cell cycle process completed and the next never occurring. Cdc14 endocycles at fixed Clb2 levels challenge this idea, as do other autonomous cell cycle oscillators (see introduction). The regular oscillation of Cdc14 localization, with frequency controlled by Clb2kd levels, suggests a regulated process rather than noisy or sporadic failure of normal control. As an alternative to ratchet models, to account for existence and entrainment of these endocycles at a common frequency, we propose that the Cdk cycle phase-locks other independent oscillators to order cell cycle events.

Such a PL model is consistent with mechanistic regulatory details that can also support ratchet models; for example, the ability of high mitotic cyclin-Cdk activity to prevent SPB 'licensing' for duplication is readily interpreted as a mechanism of oscillator coupling. The PL model may provide deeper insight into cell cycle regulation since it integrates endocycle phenotypes and normal cell cycle regulation into a coherent framework. Above, we presented experiments supporting PL over ratchet control in the free-running cell cycle, as well as in blocked cells.

Cdc5 proteolysis, controlled by a Cdc14-Cdh1-Cdc5 negative feedback loop, probably contributes to Cdc14 endocycles at high mitotic cyclin levels. Similar conclusions have been reached by others, by different methods (R. Visintin, pers. comm.). Nevertheless, Cdh1-dependent proteolysis of Cdc5 is not the only mechanism causing Cdc14 re-sequestration, since re-sequestration still happened after a delay in the absence of Cdh1; we observed G1 Cdc14 endocycles in *bub2 cdh1* cells; and the Cdh1 requirement for endocycles in Clb2kd-blocked cells was lost at very high (>2X peak) Clb2kd levels (Fig. 3B). The oscillatory Cdc14 module may include parallel mechanisms, ensuring robust oscillation. Interestingly, we found that Mob1-GFP localized to SPB periodically during Cdc14 endocycles in phase with Cdc14 release (Fig. S6B). Since Mob1's SPB localization may signal MEN activation (Stegmeier and Amon, 2004; Yoshida and Toh-e, 2001), MEN activity may oscillate to drive Cdc14 endocycle even in the absence of Cdc14-Cdh1-Cdc5 negative feedback.

The severe disruptions in normal Cdc14 release by manipulations specifically predicted to perturb the autonomous Cdc14 release module, even in free-running cell cycles, imply that oscillatory function of this module is required for normal Cdc14 release timing. A PL control system could transit evolutionarily to a pure ratchet-control system; indeed, this is formally the result of increasing coupling strength to sufficiently high values that the peripheral oscillator will not move within a physiological timescale without a 'kick' from the entraining oscillator. Different cell cycle systems, even if initially independently oscillatory, may vary in the degree to which they are at present under ratchet control; for example, DNA replication may be entirely under cyclin-Cdk ratchet control in modern eukaryotes, despite its intrinsically oscillatory character. This may represent a tradeoff between the robustness and simplicity of the PL mechanism, and the cost in some systems of occasional uncoupling (aneuploidy in the case of DNA replication).

Modular design and evolution of the cell cycle

Modularity may create functional robustness and evolvability (Bhattacharyya et al., 2006; Hartwell et al., 1999). Our study suggests that some cell cycle modules are intrinsically

oscillatory; coupling of these oscillators to the central Cdk oscillator can nevertheless readily ensure once-per-cell-cycle control (Fig. 5A,B).

The eukaryotic cell cycle evolved from a Cdk-free precursor, since Cdk is a eukaryotic-specific kinase (Nasmyth, 1995). Further, Cdks may have evolved late, after other eukaryotic cell-cycle-regulatory protein kinases (Krylov et al., 2003). If processes such as DNA replication and cell division were intrinsically oscillatory before the emergence of Cdk, then Cdk would only need to gain the ability to modulate these oscillators to gradually become a master regulator. This satisfies the evolutionary requirement of utility of intermediate forms (Darwin, 1859).

We speculate that in primordial eukaryotes, multiple autonomous biochemical oscillators entrained each other to an approximate aggregate rhythm. Cyclin-Cdk oscillation could then have evolved, yielding a stable PL structure. Selection for entrainment in large populations of simulated oscillators selects central pacemaker oscillators (Brede, 2008; Sendina-Nadal et al., 2008), which provided stability to weakening of oscillator coupling as an unselected 'phenotype'. Further, unevolved random networks of coupled oscillators, constructed computationally in a standard manner (Kuramoto, 1975), showed increased stability of spontaneous entrainment with increasing centralization of control in network generation (Fig. S6C).

The predominant model for cell cycle control involves direct cyclin-Cdk control of cell cycle events, with surveillance checkpoints to ensure order (Morgan, 2007). An earlier model (Morgan, 2007; Pringle and Hartwell, 1981), considered cell cycle control to be due to parallel, interlocking but unidirectional morphogenetic pathways arranged in a functional sequence map. Our PL model combines aspects of both: a central cyclin-Cdk oscillator phase-locks independent oscillatory modules controlling specific cell cycle events. Such independent cyclical processes are analogous to 'independent functional sequences' in the *cdc* formulation – processes that can operate freely in parallel, such as DNA replication and spindle morphogenesis (Hartwell, 1974). Phase-locking of these autonomous oscillators by a central Cdk pacemaker could have been important in early cell cycle evolution, and this mechanism may control multiple autonomous cell cycle oscillators, even in modern eukaryotes.

EXPERIMENTAL PROCEDURES

Time-lapse microscopy

Pulsed expression and release of *GAL1-CLB2kd(-GFP)* in *MET3-CDC20* blocked cells and time-lapse microscopy were previously described Drapkin et al. (in press) (Bean et al., 2006).

Image analysis

Image segmentation and quantification were described(Bean et al., 2006; Charvin et al., 2008). Cdc14 release was quantified as the coefficient of variation (CV, standard deviation divided by mean) of Cdc14-YFP pixel intensities (mother and bud treated separately), divided by the Net1-mCherry CV. A narrow band-width GFP filter (Chroma, 41020) and the

contribution of pure GFP to the YFP channel allows computational recovery of pure YFP signal. The small amount of bud-neck-localized Cdc14 was excluded from quantification. Smoothing of time-lapse series was by 3-neighbour averaging method.

Model calculations and computer simulations

Basic forms of the ODE model in Fig.3F and parameters are adopted from Chen's 2004 cell cycle model. Simulation is performed using MATLAB, as is in Fig.5B. Simulation of Kuramoto oscillator population in Fig.S6C was programmed in C language with *Numerical Recipe 2.0* package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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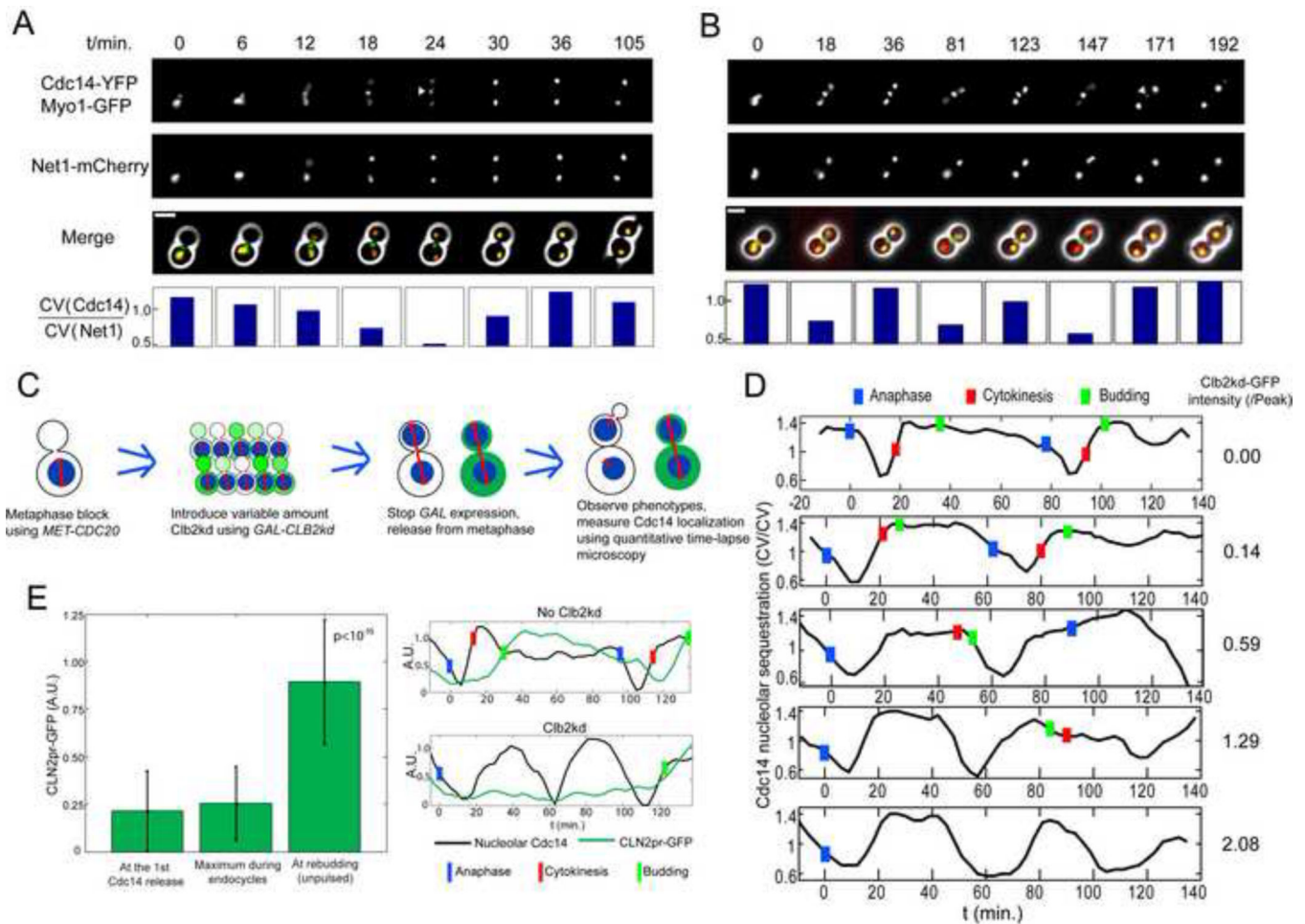
Figure 1. Cyclical Cdc14 release uncoupled from cell cycle progression

A, B. *MET3-CDC20 CDC14-YFP NET1-mCherry MYO1-GFP* cells were released from a *MET3-CDC20* block (t=0). Bottom: Cdc14 release was quantified at each time point as the following: the coefficient of variation (CV) of Cdc14-YFP signal inside a single cell, computed from fluorescent time-lapse microscopy data, is the standard deviation of YFP pixel intensity across the cell, divided by the mean intensity; CV of Cdc14-YFP is then divided by CV of Net1-mCherry, and this ratio will be high in cells with Cdc14-YFP concentrated in specific regions, and low when Cdc14-YFP is dispersed through the cell. Triangle: disappearing Myo1 ring. Scale bar: 5 microns. **A:** control. **B.** Clb2kd was pulsed for 30 min. before release. **C.** Schematic of procedure for loading cells with undegradable Clb2kd (green) before ME. Nucleus is shown in blue, spindle in red **D.** Pulsed Clb2kd-GFP was quantified (right column) in units standardized to the peak level of Clb2 attained in a normal cell cycle, and Cdc14 localization quantified (n=170). Blue bars: anaphase (nucleolar separation, marked by Net1-mCherry); Red bars: cytokinesis (Myo1 ring disappearance); Green bars: bud emergence. **E.** *CLN2* promoter expression during Cdc14 endocycles. A *CLN2pr-GFP-PEST* strain was pulsed with Clb2kd as in (B) for 35 minutes. GFP intensities at the first Cdc14 release, maximum during endocycles (n=40), and at rebudding in unpulsed control cells (P<10^{-15}. Error bars: standard deviation). See also figure S1 and movie S1.
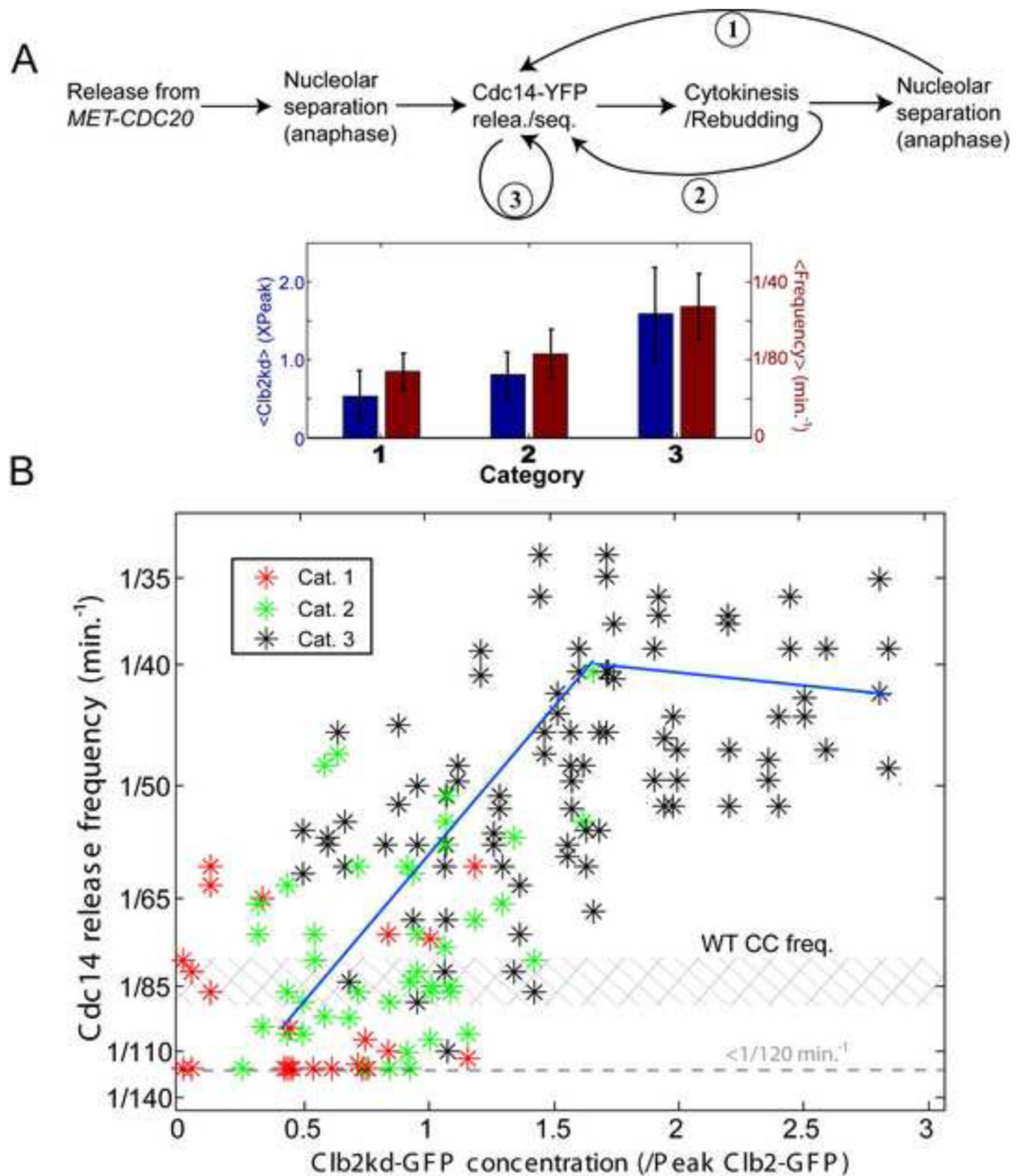
Figure 2. Clb2kd level controls the Cdc14 endocycle period
A. Trajectories for Clb2kd-pulsed cells. Category 1: essentially normal cell-cycle progression and Cdc14 release/sequestration; Category 2: a second Cdc14 release occurred between rebudding and nucleolar separation in the next cell cycle; Category 3: Cdc14 endocycles without cytokinesis or rebudding. Below: category means and standard deviations of Clb2kd-GFP concentration (blue) and Cdc14 release frequencies (red). **B.** Cdc14 release frequencies plotted against Clb2kd-GFP level for cell categories: inverse of intervals between first and second Cdc14 release (categories 1 and 2), or average

frequencies of one cell's Cdc14 endocycles (category 3; correlation between the 1st and 2nd cycle is shown in Fig. S2B). Shaded: range of cell cycle frequencies for cycling *MET3-CDC20* mother cells. 31/170 category 1; 45/170 category 2; 94/170 category 3.. See also figure S2.
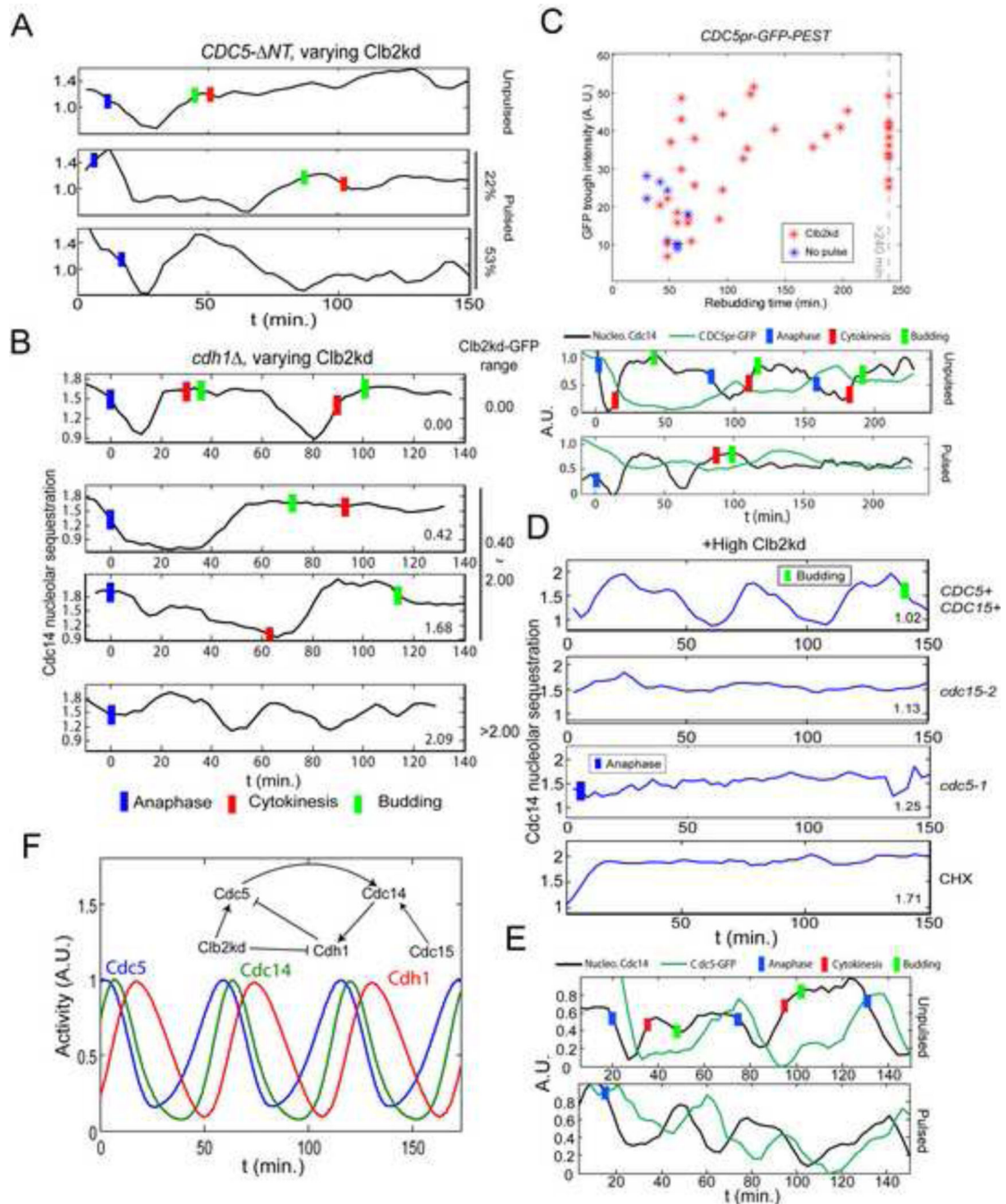
Figure 3. Requirements for Cdc14 endocycles

A. As in Fig. 1B, but cells also *cdc5::3XCDC5-ΔNT*. Among cells whose rebudding was delayed for at least 25 minutes (implying >1X peak Clb2kd, Fig. S1B), 19/36 cells showed fast Cdc14 release and resequestration, followed by prolonged Cdc14 release. 8/36 showed only prolonged Cdc14 release. **B.** As in Fig. 1D, but also *cdh1Δ*; 30 min Clb2kd-GFP pulse; typical traces for the indicated Clb2kd-GFP ranges. Among cells with >1X peak Clb2kd-GFP (n=86), 41% showed a prolonged Cdc14 release period (middle two traces); 43% (cells with highest Clb2kd-GFP) showed release endocycles with a reduced amplitude (bottom

trace); Blue bars: anaphase; Red bars: cytokinesis; Green bars: bud emergence. **C.** *CDC5pr-GFP* cells, as in Fig. 1B. Trough GFP intensities before rebudding plotted against rebudding times; rebudding delay indicates Clb2kd levels; sample traces below. **D.** Cells as in Fig. 1D, but also *cdc15-2* or *cdc5-1*; after 35 min to allow initial Cdc14 release at permissive temperature, cells were plated for time-lapse at 37°C (restrictive temperature) (t=0). CHX: as above, except that time-lapse medium contained 0.2ng/μl cycloheximide (CHX). Among cells with >1X peak Clb2kd-GFP, 18/24 *CDC15 CDC5* cells, 0/22 *cdc15-2*, 0/30 *cdc5-1* and 3/64 cells in CHX exhibited Cdc14 release endocycles. Blue bars: anaphase; Green bars: bud emergence. **E**: As in Fig. 1B, but *CDC5-GFP* cells; 30 min Clb2kd pulsed; typical traces of Cdc14 release and Cdc5-GFP levels are shown. In 36/45 Cdc14-endocycling cells, Cdc5-GFP signal oscillated out-of-phase with Cdc14 release. **F.** ODE model simulating Cdc14-Cdh1-Cdc5 negative feedback (Supplemental Methods). See also figure S3.
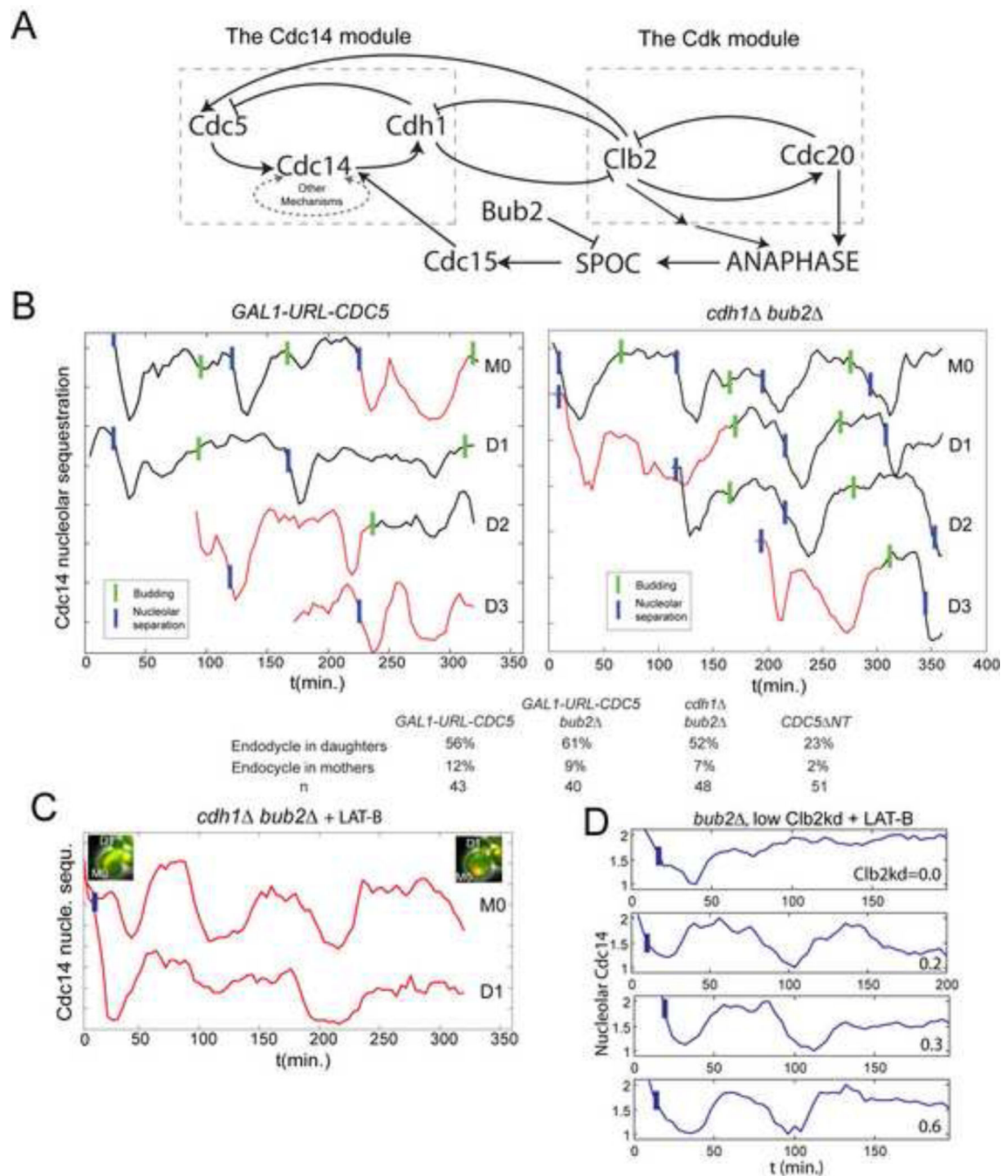
Figure 4. Cdc14 release endocycles in cycling cells or with low Clb2kd
A. Cdc14 release and Clb-Cdk control mechanisms. Left: potential autonomous Cdc14 release oscillator; right: Clb-Cdk negative-feedback oscillator. SPOC: spindle orientation checkpoint. **B.** Cdc14 release analyzed as in Fig. 1, in *bub2 cdh1* and *GAL1-URL-CDC5* cells (cycling cells, without *cdc20* block-release or Clb2kd pulse). A representative lineage (M0: mother, D1, D2...sequential daughters) exhibiting ectopic Cdc14 release endocycles (red) before bud emergence. Cdc14 release curves shifted for visualization. Below: probabilities of G1 Cdc14 endocycles. **C.** 67μM Latrunculin-B (LAT-B) was added to the

medium (t=0) to inhibit budding of a cycling *bub2 cdh1* strain. 32/44 cells demonstrated G1 Cdc14 endocycles. 14/19 daughter cells and 13/19 mother cells exhibited endocycles (maximum 4 endocycles; average=2.8). Cell images at the beginning/end of the time-course indicates no cell cycle progression. **D**. *MET-CDC20 bub2 cdc15-2* cells were arrested in metaphase at 34°C and pulsed with Clb2kd-GFP for 20 minutes (using *cdc15-2* to achieve a stable block), then released into cell cycle at 28°C with 67μM LAT-B. First anaphase (nucleolar separation) happened with normal kinetics. LAT-B effectively blocked budding, cytokinesis, and subsequent anaphase in ~90% cells. 39/46 cells containing 0.2~1.0x peak Clb2kd-GFP demonstrated Cdc14 endocycles. Four traces are shown. Blue bars: anaphase. See also figure S4.
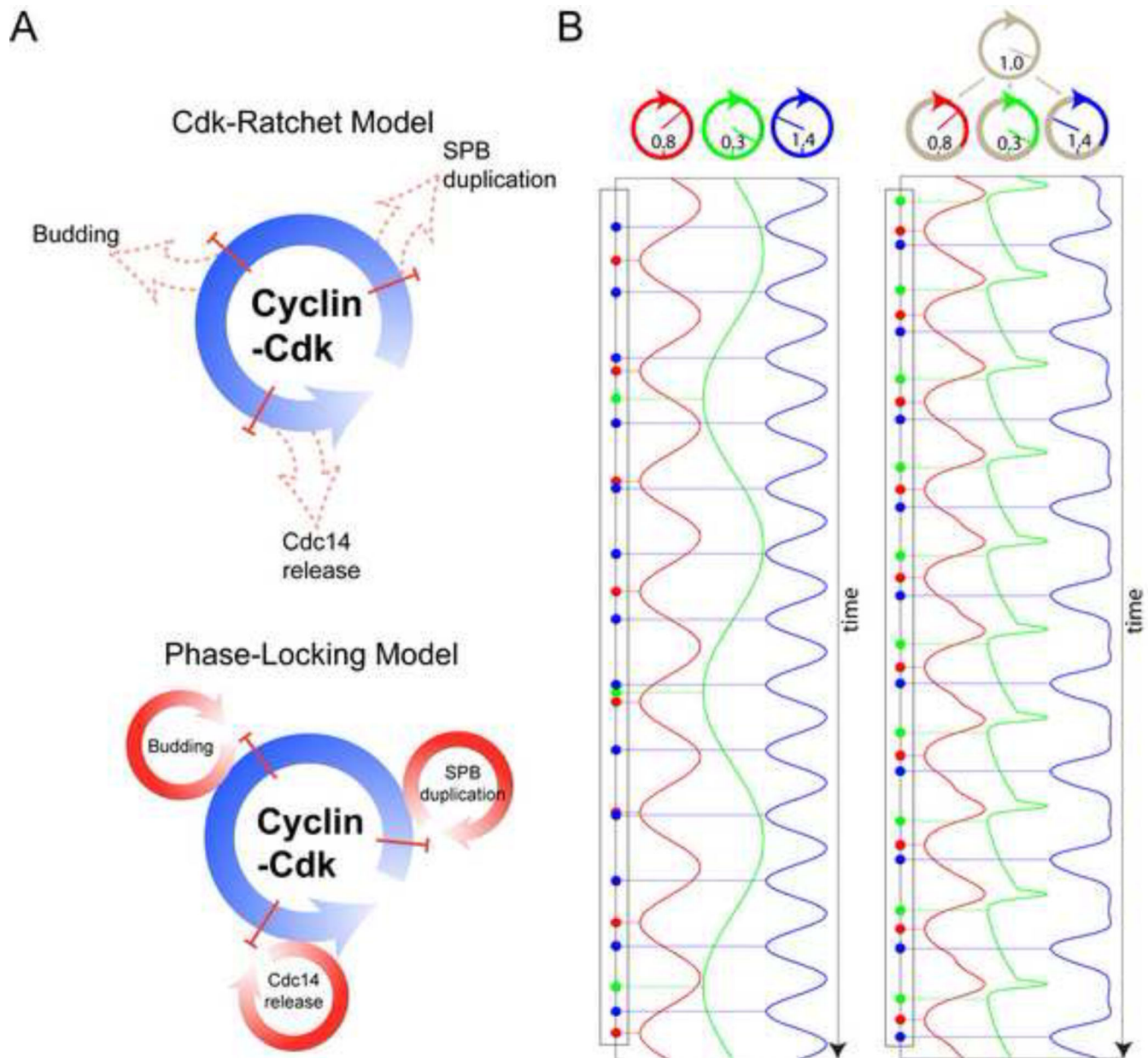
Figure 5. Cell cycle control through phase-locking
A. Schematic of ratchet (above) and phase-locking (below). **B.** Conceptual model: three simply oscillators ((Kuramoto, 1975), Supplemental Methods) with different intrinsic frequencies (indicated) control different cell cycle events. Assuming a single event is generated as the oscillator's phase evolves passing n*2π or '12 o'clock'. Without entrainment by cyclin-Cdk oscillator, each peripheral oscillator evolves uniformly, and there is no fixed order (left) among events they generate. Allowing a cyclin-Cdk oscillator to advance or delay part of the peripheral oscillators' cycles leads to phase-locking and a stable order of events (Right). See also figure S5.
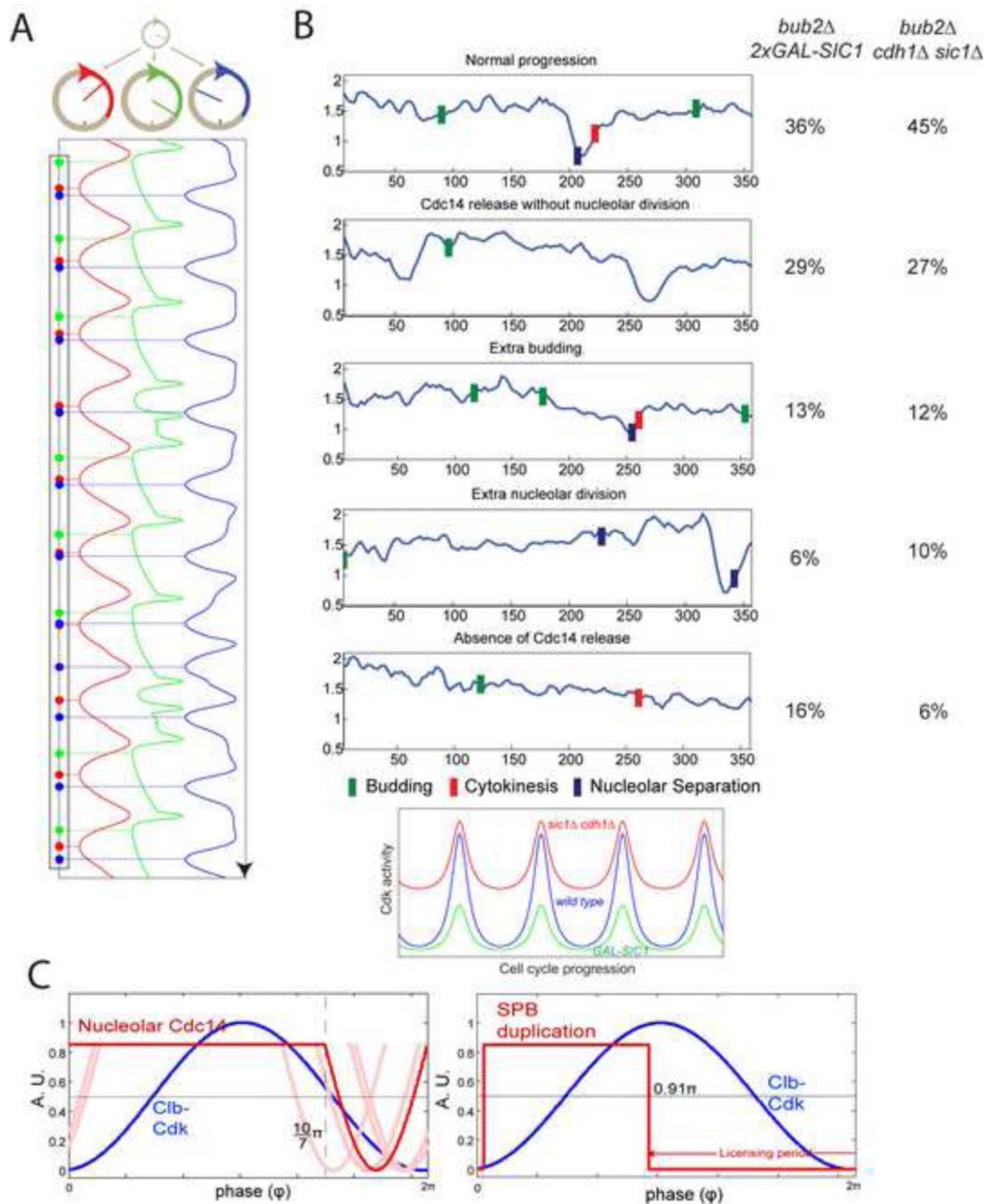
Figure 6. Experimental test of phase-locking predictions

A. Model as in Fig. 5B, but the amplitude of cyclin-Cdk oscillation is reduced by 15%, resulting in event disorder (compare Fig. 5B, right). **B.** *2XGAL1-SIC1 bub2Δ* or *cdh1Δ sic1 bub2Δ* strains may reduce the amplitude of cyclin-Cdk oscillation by lowering the peak Cdk activity or raising the trough (cartoon below). *2XGAL1-SIC1* cells (n=37) were grown in raffinose medium prior to time-lapse analysis on galactose medium to induce Sic1 (t=0). *cdh1Δ sic1Δ GALL-CDC20* cells (n=35) were imaged on glucose to turn off *GALL-CDC20*. Cells were classified according the cell cycle disorder that appeared first, shown with

representative traces (right). Blue bars: nucleolar separation; Green bars: budding; Red bars: cytokinesis. **C.** Cdc14 release and SPB duplication timing in free-running cell cycles predicted by a quantitative phase-locking model. Red: best estimation of parameters; blue: sine wave simulation of Clb-Cdk cycle. Light-red: solutions with parameters altered +/− one standard deviation for one or both of the two parameters (for two combinations no solution could be obtained due to mathematical restrictions of the model; Supplemental Methods). Right panel: phase-locking could entrain SPB duplication oscillator (red) to the correct position early in the Clb-Cdk cycle (blue) (Supplemental Methods). See also figure S6.