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Periodic Cyclin-Cdk Activity Entrains an Autonomous Cdc14 Release Oscillator

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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 resequestration 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 reinitiation (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).

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.

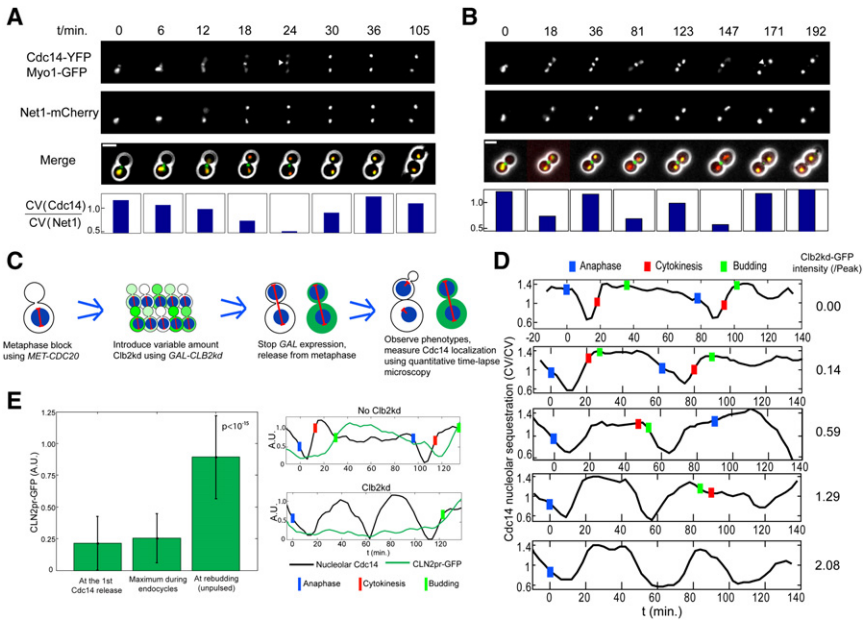

Figure 1. Cyclical Cdc14 Release Uncoupled from Cell-Cycle Progression

(A and 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. The scale bar represents 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 min. GFP intensities at the first Cdc14 release, maximum during endocycles (n = 40), and at rebudding in unpulsed control cells ($p < 10^{-15}$). Error bars represent the standard deviation. See also Figure S1 and Movie S1.

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; Figure 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 (1× peak; Figure S1A available online).

Reinducing *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 (Figure 1C).

Greater than or equal to 1X peak Clb2kd-GFP induced dose-dependent delays in cytokinesis and bud emergence (Figure 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 (Figure 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 [Figure 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 (Figure 1B,D; Movie S1).

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 (Figure 1E).

In yeast, cyclin-Cdk phosphorylation excludes the MCM helicase complex from the nucleus except in M/G1, helping to restrict prereplicative complex formation to pre-S phase (Kearsey and Cotterill, 2003). ~1X peak Clb2kd-YFP was sufficient to completely block Mcm2-GFP nuclear reaccumulation (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 (Figure S1D).

Endogenous Clb2-GFP was uniformly degraded after initial Cdc14 release, regardless of exogenously pulsed Clb2kd levels.