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Mitotic Exit: Thresholds and Targets

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Cyclin dependent kinases (CDKs) are at the heart of the cell cycle. Throughout the cycle, these complexes modify many proteins, changing various aspects of their regulation (stability, localization, etc.). As cells exit mitosis, the CDK that has driven many of the cell cycle processes is inhibited and degraded, allowing many of the kinase substrates to return to their unphosphorylated state. This assures that each subsequent cell cycle is begun in the same naïve state, again ready for CDK-dependent regulation. The studies in this thesis focus on two mechanisms by which this restoration is accomplished in the budding yeast, *Saccharomyces cerevisiae*: (1) a transcriptional program that transcribes many of the genes required for physically dividing the mother and daughter cells and beginning the next round of cell division and (2) a phosphatase that specifically removes the phosphates from sites modified by CDK during exit from mitosis.

Two transcription factors, Swi5 and Ace2, transcribe many of the genes required for physically dividing the mother and daughter cells and beginning the next round of cell division. Previously our lab has shown that locking mitotic cyclin levels, by inducing transcription of an undegradable form of the protein, causes dose-dependent delays in different cell cycle events. The first chapter addresses the contribution of the transcriptional program to this phenomenon. Interestingly, in these cells where mitotic cyclin levels were sustained, deletion of the
transcription factor Swi5 increases the mitotic cyclin inhibition, specifically as it
relates to budding and cytokinesis.

Importantly, when phosphorylated by CDK, Swi5 is excluded from the nucleus, so
in the second chapter, we investigate its localization when mitotic cyclin levels
are locked. Swi5 still enters the nucleus. In fact in some cells, Swi5 enters the
nucleus several times before the cell cycle advances. Given previous studies
from our lab showing that the release of Cdc14 phosphatase also oscillates
under these conditions, the reentry of Swi5 may support a model that a
kinase/phosphatase balance allows cell cycle progression in these cells.

All this suggests that Swi5 promotes the transcription of genes important for
promoting cytokinesis and budding despite high mitotic cyclin levels. In the third
chapter, we begin to assess the contribution of specific targets of the mitotic exit
transcriptional program to the mitotic cyclin-dependent regulation of specific cell
cycle events.

Finally, Cdc14, a phosphatase that removes the phosphate groups added by
CDKs, is sequestered for most of the cell cycle but released from the nucleolus
during the end of mitosis. In the fourth chapter, we examine the physiological
relevance of these dephosphorylation events on novel targets of the Cdc14
phosphatase.
This thesis is dedicated to my family whose love and support has been essential for its completion.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>p 1-18</td>
</tr>
<tr>
<td>Chapter One</td>
<td>p 19-35</td>
</tr>
<tr>
<td><em>Swi5 promotes budding and cytokinesis in the presence of sustained mitotic cyclin.</em></td>
<td></td>
</tr>
<tr>
<td>Chapter Two</td>
<td>p 36-57</td>
</tr>
<tr>
<td><em>Swi5 enters the nucleus despite locked mitotic cyclin levels.</em></td>
<td></td>
</tr>
<tr>
<td>Chapter Three</td>
<td>p 58-72</td>
</tr>
<tr>
<td><em>The contributions of selected Swi5 targets in promoting budding and cytokinesis.</em></td>
<td></td>
</tr>
<tr>
<td>Chapter Four</td>
<td>p 73-98</td>
</tr>
<tr>
<td><em>Identifying novel Cdc14 phosphatase targets and the physiological relevance of their dephosphorylation.</em></td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>p 99-108</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>p 109-111</td>
</tr>
<tr>
<td>References</td>
<td>p 112-128</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

**Figure Intro-1:** Schematic of protocol (courtesy of Ben Drapkin). p 15

**Figure Intro-2:** For many events of mitotic exit, locking mitotic cyclin levels causes dose-dependent delays (courtesy of Ben Drapkin). p 17

**Figure 1.1:** Swi5 promotes budding and cytokinesis in the presence of locked mitotic cyclin levels. p 24

**Figure 1.2:** In the absence of locked mitotic cyclin, the deletion of *SWI5* has minimal effects on the timing of events assayed. p 27

**Figure 1.3:** Swi5 promotes budding and cytokinesis in the presence of locked mitotic cyclin levels. p 29

**Figure 1.4:** Spindle thresholds are not affected by the deletion of *SWI5*. p 31

**Figure 1.5:** Sic1p levels are significantly lower in cells with locked mitotic cyclin. p 33

**Figure 2.1:** Swi5 nuclear entry assayed using Swi5-YFP and the nuclear marker HTB2-mCherry. p 40

**Figure 2.2:** Timing of Swi5 nuclear entry shows multiple entries during delayed exit from mitosis. p 42

**Figure 2.3:** Quantification of amount of Swi5-YFP nuclear entry shows very similar amounts of entry for both conditions. p 44

**Figure 2.4:** Cdc14 is released cyclically in cells with locked mitotic cyclins (courtesy of Ying Lu). p 48

**Figure 2.5:** Mitotic Exit may be regulated by kinase/phosphatase balance. p 50

**Figure 2.6:** Additional copies of *CDC14* rescue *CLB2kd* lethality. p 52

**Figure 2.7:** Constitutive mitotic cyclin activity shows an effect on the initial entry in timing and quantity of Ace2 localization. p 54

**Figure 3.1:** Swi5 targets identified previously by microarray (courtesy of Stefano Di Talia). p 59
Figure 3.2: Aim44 promotes budding and cytokinesis when mitotic cyclin is sustained.  p 64

Figure 3.3: Nis1 specifically promotes cytokinesis when mitotic cyclin is sustained.  p 67

Figure 3.4: Cyk3 may promote budding when mitotic cyclin is sustained.  p 69

Figure 4.1: Identification of proteins associated with released versus sequestered Cdc14 (courtesy of Joanna Bloom).  p 76

Figure 4.2: Enhanced binding of Cdc14 interactors to “substrate trapping” CDC14 mutants in vitro (courtesy of Joanna Bloom).  p 79

Figure 4.3: Enhanced binding of Cdc14 interactors to substrate trapping CDC14 mutants in vivo (courtesy of Joanna Bloom).  p 81

Figure 4.4: Confirmation of new Cdc14 substrates in vivo and in vitro (courtesy of Joanna Bloom).  p 83

Figure 4.5: The timing of the switch in formin localization is coincident with Cdc14 release.  p 88

Figure 4.6: A cdc14ΔNES strain does not degrade Clb2 during arrest.  p 91

Figure 4.7: Localization of formins during Cdc14 overexpression at a metaphase arrest.  p 92

Figure 4.8: Cells sustained in anaphase with Cdc14 release show a coincident relocalization of the formin Bnr1.  p 95
INTRODUCTION

A cell is the basic unit for all living things, and all cells are formed from existing cells. These principles make up the cell theory developed in the 1800s (Schwann and Schleiden 1847). In order to produce a new cell, the original must grow, replicate key cell components, and produce the machinery that ensures proper distribution of these components and divides it from its progeny. These processes are highly regulated and conserved across eukaryotes due to their fundamental importance to the propagation of life itself.

Some of the important components of the cell, including many organelles and proteins, are distributed randomly throughout the cell in relatively high copy numbers. Therefore, there is no need to control their distribution during cell division. However some components present in lower copy numbers, especially the genetic material, DNA, must be carefully distributed to ensure the viability of the two resulting progeny cells. A cell begins the process – called the cell cycle – with one copy of its DNA, and it must be duplicated accurately and divided with fidelity between the two resulting cells.

Chronology of the cell cycle

In further detail, the cell cycle begins with a commitment to division during a period referred to as the first gap phase (G1); in yeast, this point of commitment
is called START (Hartwell 1974) and ensures that before committing to a complete cell cycle the cell has the appropriate nutrients, size, and external cues, such as freedom from mating signals in yeast (Cross 1995).

Once the commitment to divide has been made, cells will replicate their DNA during the synthetic (S) phase. The replication begins at several sites along the cell’s chromosomes referred to as replication origins. The original DNA is carefully linked to the second copy, or sister chromatid, using a protein linker cohesin. This linkage allows orderly and equal separation of the chromatids to the two progeny cells during mitosis (see below). In addition to DNA, the duplication of another low copy-number component is required during G1 and S phases: the centrosome in animal cells or spindle pole body (SPB) in yeast. This organelle organizes the microtubules in the cell in order to form the spindle in mitosis.

Cell growth is usually continuous throughout the cell cycle (Hereford and Hartwell 1974); however, in the budding yeast the growth is not evenly distributed. At a time coincident with DNA replication, a shift in growth occurs towards a “bud” which becomes the new daughter cell. At this point, the mother stops expanding with all growth instead directed to the nascent bud site. The resulting daughter will generally be smaller than its mother at the time of cell division. A lag in the subsequent G1 in the daughter cell cycle will allow it to reach a homeostatic size.
before committing itself to the next cell cycle (Di Talia et al. 2007, Hartwell and Unger 1977, Johnston et al. 1977).

After S phase, cells pass through a second gap phase (G2) that allows for several regulatory checkpoints and then enter mitosis. Mitosis comprises (1) the division of the replicated genome into equal parts to both mother and daughter cells and (2) the physical separation of the cells (cytokinesis). During the early stages of mitosis, the spindle is formed by the separation of the two spindle pole bodies, and the chromosomes are condensed, attached to both spindle pole bodies, and prepared for separation. Unlike in animal cells, the nuclear envelope surrounding the DNA does not break down in budding yeast during mitosis; therefore, the spindle pole bodies remain embedded in the nuclear membrane. To segregate the sister chromatids, one spindle pole goes to each cell body during anaphase. Mitotic exit includes disassembly of the spindle, cytokinesis, and some preparatory steps for the subsequent cell cycle (including reloading origins of replication).

**Oscillations in CDK activity drive the cell cycle.**

This orderly progression of events during the cell cycle is primarily controlled by one enzymatic activity: the serine and threonine directed cyclin-dependent kinase (CDK). In budding yeast, a single essential CDK (Cdc28) is responsible
for driving the entire cell cycle and is homologous to Cdk1 in other organisms (Beach et al. 1982, Hartwell et al. 1974, Lee M. G. and Nurse 1987).

CDK activity is dependent on binding to its partner cyclins, originally discovered in sea urchin eggs through their periodic oscillation of protein level during the cell cycle (Evans et al. 1983). The synthesis and degradation of cyclins is tightly regulated, leading to these characteristic oscillations. During the time when they are present, cyclins pair with a CDK to phosphorylate substrates in order to systematically complete the events of the cell cycle. In addition to cyclin binding, the CDK activity in a cell depends on binding of CDK inhibitors and inhibitory phosphorylation (see below). The cell cycle begins in a low CDK activity state, but as the cell progresses, cyclins are sequentially synthesized to coordinately activate CDK.

Cyclins, CDKs, and many of the mechanisms governing their regulation of the cell cycle are highly conserved across eukaryotes; however, some details vary. Specifically in the budding yeast, nine cyclin genes interact with Cdc28 during different parts of the cell cycle, with three being termed G1 cyclins (CLN1-3) and six termed “B-type” cyclins (CLB1-6) (Bloom and Cross 2007a). Temporally, Cdc28 is first activated by the G1 cyclins to start the cell cycle, allowing the emergence of the nascent bud through polarized growth, the duplication of the spindle pole body, and the induction of later B-type cyclin expression (Cross and
Tinkelenberg 1991). Next, the S phase B-type cyclins Clb5 and Clb6 ensure efficient initiation of DNA replication (Epstein and Cross 1992, Schwob and Nasmyth 1993). Finally, the mitotic cyclins Clb1-4 are responsible for progression into mitosis, including spindle morphogenesis, the switch to isotropic growth, and shutting off the G1 cyclins (Amon et al. 1993). Typically, the cyclins are destroyed or inhibited during the transition out of mitosis (see below), returning the cell to the low CDK activity state with which it began the cycle.

Except for CLN3, the cyclins are expressed in paralogous pairs. This apparent redundancy of the cyclins is probably due to the whole genome duplication event that took place in budding yeast (Kellis et al. 2004). Because the cyclins all work through the activation of Cdc28, the need for specific cyclins is uncertain. In both budding and fission yeast, a single B-type cyclin can replicate DNA and drive mitosis: Clb2 and cdc13, respectively (Fisher and Nurse 1996, Hu and Aparicio 2005). Cells with only B-type cyclins Clb1-2 (clb3-6Δ) are inviable but can be rescued by expressing CLB2 earlier under the CLB5 promoter and removing the inhibition of early expressed Clb2 by deleting the inhibitory kinase Swe1 (Hu and Aparicio 2005). Additionally, cells can survive without all three G1 cyclins when either Clb5 is overexpressed or a stoichiometric inhibitor of B-type cyclin/CDK complexes, Sic1, is deleted (Epstein and Cross 1992, Tyers 1996).
Despite these redundancies, there are instances when a cyclin/CDK activity cannot fully substitute for that of another cyclin/CDK (Miller M. E. and Cross 2001). For instance when $CLB2$ is expressed early under the $CLB5$ promoter, DNA replication initiation is less robust (Cross \textit{et al.} 1999). This is due to differences in cyclin specificities for targets; Clb5/CDK phosphorylates a subset of its substrates through interactions with its hydrophobic patch (Cross and Jacobson 2000). By contrast, Clb2/CDK shows no highly specific substrates but instead has a higher intrinsic activity, therefore phosphorylating a broader number of targets (Loog and Morgan 2005).

**Transcriptional regulation in the cell cycle**

The cyclins are not the only cell cycle regulators under tight transcriptional control during the cell cycle. About 15\% of the budding yeast genome displays significant periodicity in transcription during the cell cycle (Spellman \textit{et al.} 1998). Periodic transcription of cell cycle genes may be a useful mechanism to enforce the order of cell cycle events, to improve the coupling of related processes, or may simply be a method of conserving resources.

There are many overlapping waves of transcriptional activation during the budding yeast cell cycle. In G1, two related transcription factors with dissimilar target sequences, Swi4-Swi6 (SBF) and Mbp1-Swi6 (MBF), coordinately activate \textasciitilde 200 genes (Koch and Nasmyth 1994, Spellman \textit{et al.} 1998). These genes
include the G1 cyclins \textit{CLN1} and \textit{CLN2}, the S phase cyclins \textit{CLB5} and \textit{CLB6}, as well as genes required for budding, DNA replication, and SPB duplication (Spellman \textit{et al}. 1998, Wittenberg and Reed 2005). Cln2 reinforces its transcription through positive feedback, which commits the cell to enter the next cell cycle (Cross and Tinkelenberg 1991, Dirick and Nasmyth 1991, Skotheim \textit{et al}. 2008).


At the end of mitosis, two groups of genes are activated, regulated by either Mcm1 or the combination of Swi5 and Ace2. (1) Mcm1 binds a palindromic sequence called an early cell cycle box (ECB) (McInerny \textit{et al}. 1997); however for most of the cell cycle this is inhibited by the presence of repressors Yox1 and Yhp1 (Darieva \textit{et al}. 2010, Pramila \textit{et al}. 2002). Mcm1 targets include \textit{SWI4} and \textit{CLN3}, important activators of G1/S transcription (Pramila \textit{et al}. 2002). (2) Swi5

While Swi5 and Ace2 bind the same sequences in vitro and share many targets, they differ in their regulation (e.g. localization, timing of nuclear entry, and interactions with co-activators and co-repressors) and are each uniquely responsible for activating a subset of targets (Dohrmann et al. 1996, Dohrmann et al. 1992). For example, Swi5 is solely responsible for the transcription of the HO endonuclease because of its unique interaction with a homeodomain protein Pho2 (Dohrmann et al. 1992, McBride et al. 1999, Nasmyth et al. 1987). Alternatively, Ace2 is restricted to the daughter nucleus by phosphorylation of its distinct nuclear export sequence by a daughter-restricted kinase and is uniquely responsible for the transcription of the chitin synthase gene CTS1 (Colman-Lerner et al. 2001, Dohrmann et al. 1992, Jansen et al. 2006, Jensen et al. 2000, Mazanka et al. 2008, Weiss et al. 2002). More detail about the regulation of these transcription factors is included in subsequent relevant chapters.

Much of this transcriptional network is regulated in part by the activity of CDK. In addition to the regulation mentioned above, in early G1, the transcription factor SBF is repressed by Whi5; phosphorylation of Whi5 by Cln3/CDK in late G1 leads to export from the nucleus and SBF activation (Costanzo et al. 2004, de Bruin et al. 2004, Jorgensen et al. 2002, Zhang J. et al. 2002). Mitotic cyclins
inactivate SBF and MBF, thus shutting off G1/S transcription (Amon et al. 1993, de Bruin et al. 2006, Siegmund and Nasmyth 1996). G2/M-specific transcription is activated by the Clb2/CDK dependent phosphorylation of Ndd1, which converts Fkh2 from a transcriptional repressor to an activator (Reynolds et al. 2003).

However, transcriptional oscillation can occur in the absence of CDK oscillation. Budding yeast with all six B-type cyclins deleted (clb1-6Δ) activate most (~70%) cell cycle regulated genes efficiently, despite their inability to replicate DNA or enter mitosis (Orlando et al. 2008). Transcription factors, in addition to activating the effectors of cell cycle events, also activate other transcription factors that drive subsequent phases, thus pushing the cell cycle forward (many examples are noted above, including Mcm1-Fkh2-Ndd1 promotion of Swi5 and Ace2 transcription) (Lee T. I. et al. 2002, Pramila et al. 2006, Simon et al. 2001). This leads to a CDK-independent persistence of transcriptional oscillation.

Mitotic exit: Inactivation of mitotic cyclins and release of Cdc14 phosphatase

As cells exit mitosis, the cyclin dependent kinase (CDK) activity that has driven many of the cell cycle processes is inactivated, allowing many of the kinase substrates to return to their dephosphorylated state.
During mitotic exit, mitotic cyclins are degraded and inhibited. Degradation occurs in two phases, both of which are dependent on the Anaphase Promoting Complex (APC) (Irniger et al. 1995, King et al. 1995). The APC is a ubiquitin ligase, which when activated by one of two differentially regulated activators (Cdc20 or Cdh1), promotes the two phases of cyclin destruction (Prinz et al. 1998, Surana et al. 1993, Yeong et al. 2000). Deleting three regions in the major mitotic cyclin Clb2 responsible for its APC-mediated destruction (two ken boxes and a destruction box) render it undegradable (referred to as CLB2kd below) (Pfleger and Kirschner 2000, Wasch and Cross 2002).

Inhibition of mitotic cyclins is achieved by both a stoichiometric inhibitor Sic1 and inhibitory phosphorylation by the Swe1 kinase (Hu and Aparicio 2005, Schwob and Nasmyth 1993, Schwob et al. 1994); however, both of these inhibitory mechanisms are themselves inhibited by mitotic cyclin/CDK activity (Jaspersen et al. 1999, Verma et al. 1997, Zachariae et al. 1998). Therefore, their inhibitory role may be restricted to cell cycle periods when the CDK activity levels are low.

In addition to the inactivation of mitotic cyclins, a counteracting serine and threonine phosphatase, Cdc14, promotes both the downregulation of CDK activity and reversal of previous CDK-dependent phosphorylations (Bloom and Cross 2007b, Visintin R. et al. 1998). Cdc14 contributes to the mechanisms described above for CDK inactivation by dephosphorylating three key substrates:
(1) Cdh1, activating the APC, (2) Swi5, a transcription factor, therefore upregulating transcription of the CDK inhibitor SIC1, and (3) Sic1 itself, preventing its ubiquitin-dependent degradation (Jaspersen et al. 1998, Schwab et al. 1997, Visintin R. et al. 1998). Cdc14 and CDK share a preference for phospho-Ser/Thr-Pro motifs, suggesting that Cdc14 may directly reverse some CDK-dependent phosphorylation events (Gray et al. 2003).

The Cdc14 phosphatase is regulated primarily by localization; it is sequestered for most of the cell cycle in the nucleolus by a scaffolding protein, Net1 (Shou et al. 1999, Traverso et al. 2001, Visintin R. et al. 1999). Two signaling pathways converge at the end of the cell cycle to release Cdc14 from the nucleolus and allow it to dephosphorylate targets: Cdc14 early anaphase release (FEAR) and Mitotic Exit Network (MEN). During early anaphase, FEAR triggers a qualitatively minor release of Cdc14, which remains nuclear, accumulates at SPBs and is not sufficient for mitotic exit (Shou et al. 1999, Stegmeier et al. 2002, Visintin R. et al. 1999, Visintin R. et al. 1998). When the daughter bound SPB reaches the bud, it allows the activation of MEN (Bardin et al. 2000), thus coupling Cdc14 release and cell cycle progression to proper spindle formation and nuclear segregation.

**How are order and timing preserved in the cell cycle?**

Due to its oscillation during the cell cycle and its role in many of the discrete steps involved, CDK activity is in a unique position to possibly enforce the

Depending on the exact levels of CDK activity required to promote mitotic entry and block mitotic exit, the normal oscillation of CDK activity could enforce the order of mitotic entry and mitotic exit (King *et al.* 1994, Morgan David O 2007, Morgan D. O. and Roberts 2002, Murray and Kirschner 1989, Stern and Nurse 1996, Zachariae and Nasmyth 1999). Specifically, if a high level of CDK activity is required for mitotic entry and mitotic exit requires a relative drop in CDK activity, then the normal oscillation of CDK activity would necessitate that mitotic entry occur before mitotic exit. This is termed a “ratchet” model referring to the restriction of motion in a single direction, and accordingly, temporal direction of cell cycle events could be maintained by the oscillation of CDK activity.

In the example of DNA replication, a ratchet model appears capable of ordering events and restricting the sequence to once and only once per cell cycle. In late
mitosis and early G1, the pre-replicative complex (pre-RC) is formed at origins of replication; this step is referred to as licensing. Initiation, or firing, of origins follows in S phase. The CDK regulation of these two steps enforces their order and ensures they occur once and only once per cell cycle. In the early cell cycle when CDK is low, pre-RCs are allowed to form on origins. Later, CDK activity inhibits the formation of pre-RCs through several mechanisms and components (Diffley et al. 1994, Nguyen et al. 2001, Piatti et al. 1995). Licensed origins, however, require CDK activity to initiate replication (Schwob et al. 1994, Tanaka et al. 2007, Zegerman and Diffley 2007). Indeed, CDK complexes localize to previously fired origins in order to block pre-RC formation and to prevent rereplication of DNA (Wilmes et al. 2004, Wuarin et al. 2002).

A ratchet model for ordering the events of mitosis by the rise and fall of CDK activity relies significantly on the quantitative levels of CDK activity required to promote mitotic entry and inhibit mitotic exit. For example, mitotic entry cannot be initiated by a CDK level lower than that required to block mitotic exit. Additionally, mitotic exit cannot be inhibited by levels above that of the entry threshold. Therefore, we set out to measure the amount of CDK activity necessary for these events to determine if a ratchet model could order the events of mitosis (see below).
Sustained mitotic cyclin causes dose-dependent delays in mitotic exit events

The requirement for mitotic cyclin activity to enter mitosis and the inhibition of exit from mitosis by overexpressed mitotic cyclin support a ratchet where the directional order is enforced by the rise and fall of CDK activity during each cell cycle (Nasmyth 1996, Stern and Nurse 1996, Zachariae and Nasmyth 1999). Through experiments led by Benjamin Drapkin (Drapkin et al. 2009), we developed a method to produce a fixed level of stable mitotic cyclin in cells and follow the effect during mitotic exit. The protocol involves arresting cells in metaphase; briefly inducing the transcription of a fluorescently tagged, stable mitotic cyclin (Clb2kd-YFP); and following individual cells during release to assay their ability to exit mitosis [Figure Intro-1]. The level of mitotic cyclin sustained during the release in individual cells was compared to the average peak mitotic cyclin observed in a synchronous cell cycle so as to put the value in a physiological context.

Using this method, a key prediction of the ratchet model was tested: the peak level of CDK activity in the cell cycle must inhibit exit from mitosis. Otherwise, the CDK level required for promoting mitotic entry cannot be higher than the level necessary for prohibiting mitotic exit because these cells all enter mitosis with at maximum the peak level of mitotic cyclin. By comparing the level of sustained mitotic cyclin in individual cells to their ability to execute events of mitotic exit,
Budding yeast cells are arrested in metaphase using Cdc20 depletion with a methionine repressible MET3-CDC20 allele in a cdc20 mutant background.

Using an introduced hormone-inducible allele, cells are pulsed with an undegradable version of the mitotic cyclin Clb2 (CLB2kd-YFP). Cells are then synchronously released by removal of methionine from the media, and their fluorescent signal (representing the amount of stable mitotic cyclin present) and their ability to complete individual events is assayed.
inhibitory concentration curves were generated [Figure Intro-2B].

Cells with mitotic cyclin levels sustained at the peak of a normal cell cycle (and significantly above) were capable of the events assayed (including spindle disassembly, cytokinesis, and rebudding [Figure Intro-2A]). We therefore conclude that a simple ratchet model of CDK activity is insufficient for ordering the events of mitosis. Further experiments, primarily by Ying Lu, showed that the CDK-opposing phosphatase Cdc14 is periodically released in these cells (Lu and Cross 2010). This led us to propose that the kinase-phosphatase balance is the important metric for cell cycle progression.

These experiments additionally revealed that maintaining physiological levels of CDK activity caused dose-dependent delays in the events of mitotic exit [Figure Intro-2B]. For example, while only twenty-five percent of cells with peak (100%) mitotic cyclin sustained can complete cytokinesis at 60 minutes after release, the number increases to fifty percent at 90 minutes. Each event had a different delay caused by the sustained mitotic cyclin. This will be discussed further in Chapter One. Additionally, cells with sustained mitotic cyclin (at levels less than 50% peak) exhibited defects in spindle assembly in the succeeding cell cycle (data not shown), suggesting an alternative requirement for CDK inhibition.
Figure Intro-2: For events of mitotic exit, locking mitotic cyclin levels causes dose-dependent delays (courtesy of Ben Drapkin). (A) Schematic representing the events assayed: spindle breakdown (blue), cytokinesis initiation (purple), cytokinesis completion (orange), and rebudding (green). (B) Thresholds (colors as in (A)) for individual events at 45, 60, and 90 minutes post-release with the pulse expressed as a percentage of the average peak Clb2 level observed in a synchronous cell cycle. Spindle disassembly not shown after 45 minutes because it reaches 100 percent by 60 minutes.
Rationale for present studies

In addition to my contributions to the above work, I have further investigated the mechanisms that set the individual inhibitory thresholds for events of mitotic exit. Many genes involved in cell cycle processes have altered expression patterns during the cell cycle to impose the order and timing of events. During mitotic exit in budding yeast, two transcription factors, Swi5 and Ace2, transcribe many of the genes required for physically dividing the mother and daughter cells and for beginning the next round of cell division (Di Talia et al. 2009, Knapp et al. 1996, Piatti et al. 1995, Toyn et al. 1997). The first three chapters of this work detail the specific contribution of Swi5 to setting the mitotic cyclin thresholds for budding and cytokinesis, how Swi5 localization is affected by sustained mitotic cyclin, and which transcriptional targets of Swi5 contribute to setting the thresholds of budding and cytokinesis.

In the fourth chapter, I detail the discovery of novel targets of the phosphatase Cdc14 during mitotic exit. While its role in inhibiting CDK activity has been well studied, its role in resetting other CDK-phosphorylated proteins is less well understood. In collaboration with the laboratory of Brian Chait and a postdoctoral fellow in our laboratory, Joanna Bloom, several targets were identified, and here, I present preliminary evidence that the Cdc14-dependent dephosphorylation of a protein involved in cytokinesis leads to a change in its localization.
CHAPTER ONE

Swi5 promotes budding and cytokinesis in the presence of sustained mitotic cyclin.

INTRODUCTION

Our studies began with an interest in determining the amount of CDK activity required to inhibit mitotic exit. Previous experiments showed that gross overexpression of mitotic cyclin arrests cells in telophase (Surana et al. 1993), but a CDK ratchet model (see Introduction) requires inhibition at physiological CDK levels. In order to test the ratchet model for ordering the events in mitosis, we developed a protocol for determining the effect of maintaining physiological levels of CDK activity after entry to mitosis (Drapkin et al. 2009).

The protocol [Figure Intro-1], developed primarily by Ben Drapkin, involved arresting cells in metaphase using depletion of the APC activator Cdc20 (Hartwell and Smith 1985, Irniger et al. 1995, Lim et al. 1998, Sethi et al. 1991, Wasch and Cross 2002). A pulse of fluorescently tagged, undegradable mitotic cyclin (Clb2kd-YFP) was induced from the GAL1 promoter using an exogenous hormone (deoxycorticosterone) in cells constitutively expressing a Gal4-rMR fusion (Picard 2000). Cells were then released from the metaphase block, and individual cells were assayed for the amount of stable mitotic cyclin they received from the pulse and their ability to complete cell cycle events within the assayed
time period (including spindle disassembly, cytokinesis, and new bud formation [Figure Intro-2B]).

The average peak of a fluorescently tagged mitotic cyclin (Clb2-YFP) under its endogenous promoter in a synchronous cell cycle was used to standardize the measurements of stable mitotic cyclin (Clb2kd-YFP). This provided a physiological reference for our measurements. Previous estimates have placed estimated this peak value at around 3,000 molecules of mitotic cyclin Clb2 per cell (Cross et al. 2002). During a normal cell cycle, a cell will only be exposed to approximately the peak amount of cyclin and below. However, activation of the spindle checkpoint inhibits Cdc20 (Hwang et al. 1998), causing an accumulation of mitotic cyclin two fold higher than the peak (Drapkin et al. 2009). Therefore, we consider that ranges up to twice our defined peak may be physiologically relevant to cells in certain contexts.

When the level of sustained mitotic cyclin in individual cells is compared to a cell’s ability to accomplish a mitotic event (at a given time after release), the curves generated showed sharp inhibitory thresholds for the events of mitotic exit [Figure Intro-2B]. Rather than an all-or-none threshold, stable mitotic cyclin generated process-specific, dose-dependent delays. For example, spindle disassembly, while blocked by overexpression of mitotic cyclin (Surana et al. 1993), was only delayed by ~15 minutes when a physiological level of mitotic
cyclin (100% peak) was sustained. In cells with twofold peak levels of mitotic cyclin, spindles were sustained for 45 minutes, while no physiological level of mitotic cyclin could sustain spindles for more than 60 minutes (data not shown). This trend of dose dependent delays was also observed for the initiation of bud ring contraction, completion of cytokinesis, and formation of a new bud [Figure Intro-2B]. Overall, inhibiting mitotic exit required levels of mitotic cyclin well above those achievable during a normal cell cycle.

The mitotic cyclin inhibitory thresholds for individual mitotic events could enforce their order during a normal cell cycle. The delays caused by sustained mitotic cyclin were process specific, and generally, the same amount of cyclin inhibited later events more robustly [Figure Intro-2B]. This was further supported by the observation that a portion of cells with sustained mitotic cyclin rebudded before completing cytokinesis. The disordering of these events could be explained by the observation that the budding threshold shifts faster than the threshold for cytokinesis [Figure Intro-2B].

To ascertain the molecular determinants of the mitotic cyclin inhibitory thresholds, I tested the role of the primary transcriptional activator during the transition from mitosis to the subsequent cell cycle, Swi5. During the exit from mitosis and the early stages of the next cell cycle, the mitotic cyclin levels usually drop and Cdc14 phosphatase is released from its nucleolar anchor (Shou et al. 1999).
When Cdc14 dephosphorylates Swi5 (Visintin R. et al. 1998), it activates a coordinated transcriptional network to prepare for the important transition from the end of one cell cycle to the beginning of another.

\textit{SWI5} is transcribed starting in G2 and present for the entirety of mitosis (Althoefer et al. 1995, Lydall et al. 1991). However, Swi5 is excluded from the nucleus by CDK-dependent phosphorylation. Following the release of Cdc14 phosphatase in anaphase, Swi5 dephosphorylation uncovers a nuclear localization sequence (NLS), permitting its entry into the nucleus (Moll et al. 1991, Nasmyth et al. 1990).

In the nucleus, Swi5 is able to bind to promoters and recruit chromatin-remodeling factors (Cosma et al. 1999, Cosma et al. 2001). While first characterized for its role at the promoter of the HO endonuclease required for mating-type switching in yeast, Swi5 has since been shown to be important for the transcription of many more genes, including the stoichiometric inhibitor of mitotic cyclin/CDK complexes Sic1 (Knapp et al. 1996, Toyn et al. 1997). More detail about Swi5 transcriptional targets will be discussed in Chapter Three.

In early G1, Swi5 is phosphorylated, allowing it to be recognized by a ubiquitin ligase complex (SCF-Cdc4) and degraded by the proteasome (Kishi et al. 2008). The kinase responsible has yet to be identified; however, two non-essential
CDKs have been suggested to contribute to the instability of Swi5: Srb10 and Pho85 (Kishi et al. 2008, Measday et al. 2000). Due to tight regulation of its nuclear entry and stability, Swi5 is only active during a brief window of the cell cycle immediately following anaphase.

RESULTS
To determine the role of the mitotic exit transcriptional network in setting the mitotic cyclin inhibitory thresholds for individual mitotic exit events, I compared a wild type (WT) strain with one that has the \textit{SWI5} gene deleted (\textit{swi5}\text{Δ}). The protocol is otherwise the same as the one used in previous experiments [Figure Intro-1] (Drapkin et al. 2009). At timepoints following release from the metaphase arrest, samples were taken, and individual cells assessed for (1) the amount of stable mitotic cyclin induced and (2) the ability to accomplish individual mitotic exit events [Figure 1.1A]. Using fluorescently labeled tubulin (\textit{TUB1-CFP}), spindle disassembly was monitored. The shrinking and disappearance of the actomyosin ring (\textit{MYO1-mCherry}) marked cytokinesis initiation and completion, respectively. The formation of a new bud was scored by the establishment of a new actomyosin ring. The amount of mitotic cyclin is standardized to the peak (defined as 100\% or 1\x) amount of Clb2 reached in a synchronous cell cycle, as described. This allows us to establish that the levels assayed are physiologically relevant.
Figure 1.1: Swi5 promotes budding and cytokinesis in the presence of locked mitotic cyclin levels. (A) Representative cells, 45 minutes after release. Merged Tub1-CFP and Myo1-mCherry on left; Clb2.kd-YFP on right. WT (left, blue) and swi5Δ (right, red). (B) Thresholds for each event at 45, 60, and 90 minutes after release. The percentage of cells that complete an event is plotted against the cyclin concentration, expressed as a fraction of the peak from a synchronous cell cycle. The data were binned to ensure a sufficient number of cells within a given range of mitotic cyclin levels. In cells with SWI5 deleted, a similar level of sustained mitotic cyclin causes a stronger delay or inhibition of cytokinesis and rebudding than in wild type.
Figure 1.1

A 45' post release: **TUB1-CFP**  **MYO1-mCherry**  **CLB2,kd-YFP**

WT  swi5Δ

B

<table>
<thead>
<tr>
<th></th>
<th>45'</th>
<th>60'</th>
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<tbody>
<tr>
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<td><img src="image5" alt="Graphs" /></td>
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<td><strong>Rebudding</strong></td>
<td><img src="image7" alt="Graphs" /></td>
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[CLB2,kd-YFP]/[peak]
In cells that were not induced with a pulse of stable mitotic cyclin, the deletion of SWI5 had minimal effects on the events assayed [Figure 1.2]. When cells were loaded with the stable mitotic cyclin and released, the same dose-dependent delays were seen in wild type cells that we observed previously [Figure1.1B]. In swi5Δ mutant cells, the same amount of mitotic cyclin had a more severe inhibitory effect on initiating ring contraction, completing cytokinesis, and rebudding in the subsequent cell cycle [Figure1.1B]. For all three mitotic exit events, the swi5Δ cells were less likely to have completed the event than a wild type cell with a similar amount of locked mitotic cyclin. This leads to the conclusion that in cells with sustained mitotic cyclin, Swi5 is promoting budding and cytokinesis. When the experiment was repeated using timelapse microscopy to follow the release with higher resolution (every 3 minutes) and for longer (360 minutes), the result was reproduced [Figure 1.3].

Since one of the transcriptional targets of Swi5 is Sic1, the stoichiometric inhibitor of mitotic cyclin/CDK complexes (Toyn *et al.* 1997), deletion of SWI5 may be decreasing the inhibition of the mitotic cyclin during the timecourse. While expression of a stable mitotic cyclin (Clb2kd) from its endogenous promoter is lethal, the strain can be rescued by overexpression of Sic1 (Archambault *et al.* 2003, Wasch and Cross 2002). Similarly, ten copies of the endogenous SIC1 can bypass the necessity of APC-mediated degradation of mitotic cyclin altogether.
Figure 1.2: In the absence of locked mitotic cyclin, the deletion of *SWI5* has minimal effects on the timing of events assayed. The timing of cytokinesis initiation, completion, and rebudding is similar between wild type (blue) and *swi5Δ* (red).
Figure 1.3: Swi5 promotes budding and cytokinesis in the presence of locked mitotic cyclin levels. Experimental protocol is the same as in Figure 1.1, but cells were assayed after release using timelapse microscopy with three-minute resolution. Data from timelapse movies shown in scatter-plots, of wild type (blue) and \textit{swi5Δ} (red), with mitotic cyclin pulse concentration versus time after release (in minutes) for individual cells to initiate cytokinesis (top), complete cytokinesis (center), or rebud (bottom). Dashed line represents the end of the movie at six hours; points above this line are therefore cells that did not accomplish the event before the end of the movie. Results from a Mann-Whitney test between the wild type and \textit{swi5Δ} strains are indicated by boxes (approximating bin sizes horizontally) above the graphs (\textit{p}-value given and represented by shade, with color denoting which of the two strains has the longer median time in a given bin).
Figure 1.3
(Thornton and Toczyski 2003). However, several points of evidence suggest that the effect of the *SWI5* deletion on these mitotic cyclin inhibitory thresholds is not due to a reduction in Sic1 transcription.

First, the shift in thresholds was specific to the event of cytokinesis and rebudding; the deletion of *SWI5* had no effect on the disassembly of anaphase spindles when compared to wild type [Figure 1.4A]. If the effect of the *SWI5* deletion were due to a general increase in the CDK activity, it would be expected that all thresholds would be affected. Instead, the limited effect of Swi5 argues that it specifically contributes to the ability of cells with locked mitotic cyclin to initiate and complete cytokinesis and bud in the next cell cycle. However, the possibility that the mitotic cyclin inhibitory threshold for spindle disassembly is steeper than those for cytokinesis and rebudding, obscuring an effect, cannot be eliminated.

Since *SIC1* is the Swi5 target most likely to increase general CDK activity, we further tested its ability to affect mitotic cyclin inhibitory thresholds. Drapkin *et al* (2009) investigated whether increasing the level of Sic1 affected the thresholds. If Sic1 does set the thresholds through its stoichiometric inhibition of mitotic cyclin/CDK complexes, then it would be predicted that doubling the amount of Sic1 would require a compensatory doubling of the cyclin for the same delay in mitotic exit events. Doubling the *SIC1* gene dosage (which resulted in twice
Figure 1.4: Spindle thresholds are not affected by the deletion of \textit{SWI5}.

\textbf{(A)} Threshold for anaphase spindle disassembly (shown at 45 minutes post-release) for wild type (blue) and \textit{swi5}\textsuperscript{Δ} (red) is not affected by the deletion of \textit{SWI5}.  \textbf{(B)} Timing of spindle disassembly is similar for wild type and \textit{swi5}\textsuperscript{Δ} strains when unpulsed (solid lines) or with locked mitotic cyclin (dashed lines).
the mRNA levels) did not change the thresholds; however, increasing it six fold shifted them moderately (Drapkin et al. 2009). Furthermore, when in vitro histone H1 kinase activity associated with the stable mitotic cyclin pulse was assayed throughout the timecourse, the activity of the induced undegradable protein in both the wild type and 2xSIC1 remained stable. Together, these results indicate that Sic1 is not setting the thresholds through inhibiting mitotic cyclin complexes.

Sic1 levels are periodic due to CDK-mediated proteolysis and CDK-inhibited periodic transcription (Verma et al. 1997, Knapp et al. 1996, Schwob et al. 1994). We hypothesized that any Sic1 produced may be quickly turned over when mitotic cyclin levels are sustained. I tested this by measuring the protein level of Sic1. When cells were released from metaphase, Sic1 was significantly decreased in cells that had sustained mitotic cyclin compared to those that were unpulsed [Figure 1.5A]. When compared directly, the peak of Sic1 protein was decreased by half in cells with sustained mitotic cyclin [Figure 1.5B].

Together, the inability of SIC1 to shift the thresholds when doubled and the lowered Sic1 protein levels in cells with sustained mitotic cyclin led us to conclude that Sic1 is not setting the thresholds through inhibition of the mitotic cyclin/CDK complexes. Assaying mitotic cyclin-associated kinase activity in a swi5Δ mutant with sustained mitotic cyclin could be used to demonstrate if there is a general increase in CDK activity in these cells due to targets besides SIC1.
Figure 1.5: Sic1p levels are significantly lower in cells with locked mitotic cyclin. (A) Protein samples collected from a wild type strain (with \textit{SIC1-GFP}) after release from metaphase in either unpulsed or pulsed (\textit{CLB2,kd}) conditions. In both treatments, SIC1-GFP peaks at 40 minutes. (B) Peak samples (40min) from same experiment in (A) compared adjacent to a peak Clb2-YFP sample. Quantification (below) was calculated by standardizing to loading control (Pgk1) and normalizing to peak Sic1-GFP from unpulsed cells.
However, previous assays of mitotic cyclin-associated kinase activity when near-peak levels of mitotic cyclin were sustained showed that the measurable kinase activity per unit protein was maximal (Drapkin et al. 2009); therefore, it is unlikely a significant increase in kinase activity will occur in swi5Δ mutants. This evidence supports the conclusion that Swi5 is specifically able to promote budding and cytokinesis despite locked mitotic cyclins, through a target other than Sic1.

CONCLUSION

Sustaining mitotic cyclins after release from metaphase does not block mitotic exit events but causes dose-dependent delays (Drapkin et al. 2009). In order to accomplish this feat, the cells depend on Swi5 to promote cytokinesis and new bud formation. Sic1, despite being a target of Swi5 and an inhibitor of mitotic cyclin/CDKs, does not have a major role in setting the thresholds, ostensibly due to its CDK-induced destruction. Because it activates an entire transcriptional program, Swi5 is in a unique position to orchestrate several events of mitotic exit. Similar regulators may help us understand the thresholds for events other than budding and cytokinesis.

This result raises several questions. First, CDK-dependent phosphorylation would be expected to maintain Swi5 in the cytoplasm (Moll et al. 1991). However, the significant effect of deleting SWI5 on mitotic cyclin inhibitory
thresholds indicates that it has an activity despite sustained CDK activity. This may be a new role for Swi5 independent of its ability to promote transcription, or Swi5 may overcome the high level of mitotic cyclin/CDK activity to enter the nucleus. In Chapter Two, I determine the localization of Swi5 in the presence of sustained mitotic cyclin levels.

Second, if Swi5 is acting in its role as a transcription factor, then we can ask if individual targets of Swi5 are responsible for setting the mitotic cyclin inhibitory thresholds for budding and cytokinesis. In Chapter Three, we assay the effect of deletions in individual Swi5 targets on these thresholds to further our understanding of the coordinated effect of the transcriptional network on mitotic exit events.
CHAPTER TWO

Swi5 enters the nucleus despite locked mitotic cyclin levels.

INTRODUCTION

The mitotic exit transcription factor Swi5 helps promote budding and cytokinesis in the presence of locked mitotic cyclins. However, a major question raised by this result is whether Swi5 is capable of entering the nucleus to promote transcription of its targets in the presence of continued CDK activity.

As discussed in Chapter One, localization of Swi5 is regulated during the cell cycle. Until anaphase, the mitotic cyclin/CDK maintains Swi5 in a phosphorylated state on three serine residues (Moll et al. 1991, Surana et al. 1991). These post-translational modifications are within or proximal to a classical bipartite nuclear localization signal (NLS) in the carboxy-terminus of the Swi5 protein (Hahn et al. 2008, Moll et al. 1991). Mutation of these serine residues to alanine, mimicking constitutive dephosphorylation, causes continuous nuclear localization of Swi5 (Moll et al. 1991).

When the phosphatase Cdc14 is released from the nucleolus in anaphase, it removes the inhibitory phosphorylations on Swi5, enabling the recognition of the NLS (Moll et al. 1991, Nasmyth et al. 1990, Visintin R. et al. 1998). Once in the nucleus, Swi5 binds to promoters and recruits chromatin-remodeling factors
(Cosma et al. 1999, Cosma et al. 2001). This allows for a coordinated transcription of approximately 30 genes (Morgan David O 2007, Spellman et al. 1998).

Ace2 is a homologue of Swi5 that has many similarities in its cell cycle regulation but some key differences. Both Swi5 and Ace2 are transcribed in G2, retained in the cytoplasm until anaphase, and finally translocated into the nucleus during mitotic exit (Dohrmann et al. 1992, Nasmyth et al. 1990). Entry into the nucleus is similarly inhibited by CDK-dependent phosphorylations and presumably permitted by Cdc14 phosphatase activity (O'Conallain et al. 1999, Sbia et al. 2008). Unlike Swi5, however, Ace2 is primarily restricted to the daughter nucleus. This localization is due to a Cbk1-dependent phosphorylation of Ace2 in a nuclear export sequence (NES) that prohibits its interaction with the export machinery (Jensen et al. 2000, Mazanka et al. 2008). Cbk1 is restricted to the daughter (Colman-Lerner et al. 2001, Jansen et al. 2006, Weiss et al. 2002). Ace2 nuclear entry and transcription is also somewhat delayed compared to that of Swi5 (Di Talia et al. 2009, Sbia et al. 2008, Toyn et al. 1997).

While the entry of both Swi5 and Ace2 into the nucleus is similarly regulated, they have different fates after entering. While Swi5 is very unstable after nuclear entry (Kishi et al. 2008, Measday et al. 2000), Ace2 levels are constant throughout the cell cycle (Sbia et al. 2008). Instead, a mechanism involving its cytoplasmic
retention promoted by G1 cyclin/CDK activity is responsible for inactivating Ace2 (Mazanka and Weiss 2010).

RESULTS

To assess the ability of Swi5 to enter the nucleus in the presence of locked mitotic cyclins, a strain with fluorescently tagged Swi5 (Swi5-YFP) and a histone (Htb2-mCherry) to mark the nucleus was used. Similar to the protocol in Figure Intro-1, cells were (1) arrested in metaphase, (2) induced with a pulse of an unlabeled, undegradable mitotic cyclin (CLB2kd), and (3) followed during release by timelapse microscopy. The cells were imaged every three minutes [Figure 2.1A]. Anaphase was scored based on the nuclear segregation of the histone marker. To quantify Swi5 nuclear entry, the ratio of the YFP signal in the nucleus (defined by HTB2-mCherry) and the cytoplasm (cell excluding nucleus) was used. Typical traces for an unpulsed and pulsed cell are shown in Figure 2.1B.

In cells without sustained mitotic cyclin (unpulsed), Swi5 enters the two nuclei (mother and daughter) soon after anaphase [Figure 2.1A and Figure 2.2A]. Surprisingly, in the cells with sustained mitotic cyclins (pulsed), Swi5 entered the nucleus with no significant delay [Figure 2.1A and 2.2A], even though other mitotic exit events were delayed. Therefore, the timing of initial entry of Swi5 is not affected by sustained mitotic cyclin.
Figure 2.1: Swi5 nuclear entry assayed using Swi5-YFP and the nuclear marker HTB2-mCherry. (A) Representative images from timelapse movies of unpulsed and pulsed conditions show similar initial nuclear entry of Swi5. Images are of first frame (left), first Swi5 nuclear entry (center), and second Swi5 nuclear entry (right); timing is from anaphase (in minutes). (B) Ratio of Swi5-YFP nuclear (defined by HTB2-mCherry) to cytoplasmic fluorescence used to determine timing and quantity of entry. Examples of traces of two cells from movies, indicated by white arrows in (A).
Figure 2.1

A

Unpulsed

-12 min (from anaphase)  3 min  108 min

Pulsed

-12 min (from anaphase)  3 min  66 min

B

unpulsed

pulsed

Nuclear to Cytoplasmic Ratio of Swl5-YFP

Time (in minutes) from movie start
Figure 2.2: Timing of Swi5 nuclear entry shows multiple entries during delayed exit from mitosis. (A) Timing of first Swi5-YFP nuclear entry in minutes from anaphase (box defines lower and upper quartiles, whiskers define minimum and maximum values, dash defines median) shows no difference between unpulsed (n=39) and pulsed (n=66) cells (with a population average of ~1x peak). (B) Timing of second Swi5-YFP nuclear entry in minutes from first anaphase shows tendency to have a shorter period in pulsed (n=38) cells despite other events being delayed compared to unpulsed (n=23). This data excludes cells that did not have a second entry during the length of the movie (6 hours).
Figure 2.2

A

Timing of first Swi5-YFP nuclear entry

Time (minutes) from anaphase

unpulsed  pulsed

B

Timing of second Swi5-YFP nuclear entry

Time (minutes) from anaphase

unpulsed  pulsed
Also, the amount of Swi5 that enters the nucleus was the same with or without sustained mitotic cyclin [Figure 2.3]. Using the nuclear to cytoplasmic ratio as a quantitative metric, the first and second entries were similar for both unpulsed and pulsed conditions. In both conditions the quantity of the first entry was larger compared to the second; however, this could be due to the preceding arrest (i.e. the amount of the protein accumulated or the maturation of the fluorophore during the arrest).

Despite these similarities, a surprising difference in the timing of later entries of Swi5 was observed. Cells without sustained mitotic cyclin completed division and progressed into the subsequent cell cycle, and Swi5 entered the nucleus immediately following the second anaphase [Figure 2.1A and 2.2B]. However in cells with locked mitotic cyclins, the timing of the next Swi5 entry was quite variable. While some cells went through their delayed cell cycle and followed a pattern similar to unpulsed cells, others had an earlier second entry of Swi5 without budding [Figure 2.1B]. This is reflected in a decrease in the median timing of the second entry [Figure 2.2B], despite the fact that most other events in these cells were delayed. In fact, some cells even had additional entries before cell cycle progression.

The repeated entries of Swi5 before cell cycle progression may be dependent on the dose of mitotic cyclin. Table 2.1 shows the average estimated level of
Figure 2.3: Quantification of amount of Swi5-YFP nuclear entry (based on nuclear to cytoplasmic ratio) shows very similar amounts of entry for both conditions. Using the ratio of the YFP signal in the nucleus to that in the cytoplasm, the amount of Swi5 entering during the first and second nuclear entries was estimated. Displayed is an average of the peak of this ratio for both unpulsed and pulsed cells (same experiment as shown in Figure 2.2).
mitotic cyclin in cells with each pattern of Swi5 nuclear entry. Because the mitotic cyclin was not fluorescently labeled in these experiments, the phenotype of the cell was used to estimate the level (see Materials and Methods). An increase in the amount of mitotic cyclin correlates with an increase in the number of times Swi5 enters before the cell cycle progresses.

Table 2.1:

Correlation between locked mitotic cyclin and Swi5 nuclear entries.

<table>
<thead>
<tr>
<th>Number of Swi5 nuclear entries before budding</th>
<th>[Mitotic cyclin]/[peak] ± standard deviation</th>
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<tbody>
<tr>
<td>1</td>
<td>0.5 ± 0.5</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>2.5 ± 1.1</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>3.1 ± 0.3</td>
<td>5</td>
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The correlation could be because overcoming an increase in the mitotic cyclin requires multiple rounds of Swi5-dependent transcription. Alternatively, it may be because the dose-dependent delay in cell cycle progression uncovers another cyclical process. However, the time between the last entry of Swi5 to bud emergence is similar in both pulsed and unpulsed cells (average ± standard deviation: 55 ± 26 versus 59 ± 22 minutes). This correlation suggests that Swi5 may play an active role in cell cycle progression but does not rule out roles for other contributing factors.
The effect of Swi5 on the thresholds of budding and cytokinesis [Figure 1.2], and its status as a target of mitotic cyclin/CDK and Cdc14 phosphatase activities lends support to a model we proposed for the mechanism of cell cycle progression in the presence of locked mitotic cyclins. Studies from our lab show that when mitotic cyclin levels are sustained during mitotic exit, an intrinsic oscillation of Cdc14 nucleolar release and resequestration is revealed [Figure 2.4B] (Drapkin et al. 2009, Lu and Cross 2010). The level of mitotic cyclin/CDK activity regulates the frequency of the release oscillations; higher levels of mitotic cyclin induce faster cycles of Cdc14 release. However, the amount and duration of Cdc14’s release is unaffected.

This finding led us to propose a model where progression of the cell cycle in the presence of locked mitotic cyclin may occur due to a shift in the kinase/phosphatase balance, especially between CDK and Cdc14 activities (Cdc14 is released, and thus activated, multiple times in the presence of sustained CDK activity), resulting in the dephosphorylation of key mutual targets despite the maintenance of mitotic cyclin activity [Figure 2.5]. The results described here for the mutual CDK/Cdc14 target Swi5 are highly consistent with this model. In cells delayed in cell cycle progression, the amount of sustained mitotic cyclin required to see oscillatory behavior in Swi5 [Table 2.1] was similar to the level necessary to see repeated release of Cdc14 without cell cycle progression (Lu and Cross 2010).
Figure 2.4: Cdc14 is released cyclically in cells with locked mitotic cyclins (courtesy of Ying Lu). (A) Cdc14-YFP release from the nucleolus (Net1-mCherry) observed by timelapse microscopy in cells with sustained mitotic cyclins. Images from a metaphase blocked cell (t = 0 min) and a cell undergoing release of Cdc14 (at t = 24 min). Arrows indicate Myo1-GFP at bud neck. (B) Cdc14 localization traces after release from metaphase block for five cells with varying amounts of sustained mitotic cyclin (column at right). Quantification is the ratio of coefficients of variation (CV=standard deviation/mean) of pixel intensities for Cdc14-YFP and Net1-mCherry. This ratio will be high in cells with Cdc14 concentrated in specific regions and low in cells with Cdc14 evenly distributed in the cells. Marked on the traces are events in mitotic exit: anaphase (blue bar), cytokinesis (red bar), and budding (green bar). Frequency of Cdc14 oscillation is increased with increasing mitotic cyclins, saturating at once per 45 minutes; however, amplitude and duration are not affected.
Figure 2.4

A

Net1-mCherry  Cdc14-YFP  Myo1-GFP  Merge

In  (0 min)

Out  (24 min)

B

Anaphase  Cytokinesis  Budding  Clb2kd-GFP intensity (/Peak)

Cdc14 nucleolar sequestration

Cdc14 nucleolar sequestration (CV/CV)

Adapted from Drapkin, et al, (2009) and Lu and Cross (2010)
Figure 2.5: Mitotic Exit may be regulated by kinase/phosphatase balance.

(A) Model for co-regulation of mitotic exit by Cdc14 and mitotic cyclins (Clb2). The relative balance of these two may be the relevant variable for ordering the events of mitosis. 

(B) In a normal cell cycle (left), kinase activity (blue) falls after anaphase (dashed line), and Cdc14 phosphatase activity increases (yellow), allowing a net decrease in the phosphorylation of targets (red). When the net phosphorylation of targets (e.g. Swi5) drops below a certain threshold (grey box), an event is triggered (e.g. Swi5 nuclear entry, budding, and cytokinesis). In the presence of stable mitotic cyclins (right), Cdc14 release may reach the threshold needed to permit mitotic exit events despite the maintenance of kinase activity.
Figure 2.5

A

Multiple inputs

Cdc14

Clb2

Sic1, Cdh1

Mitotic exit

B

Normal cycle

Pulsed Clb2kd

+ P

- P

Drapkin, et al. (2009)
To test this model, I assessed if extra copies of \textit{CDC14} could rescue the viability of a \textit{CLB2kd/CLB2} strain; heterozygous \textit{CLB2kd} expression under the \textit{CLB2} promoter is lethal due to nonphysiological accumulation of mitotic cyclin activity (Wasch and Cross 2002). This strain can be maintained by overexpression of \textit{SIC1}, which inhibits mitotic cyclin/CDK complexes, from a galactose inducible promoter. The strain was transformed with an empty vector or a centromeric or integrating plasmid with \textit{CDC14} under its endogenous promoter. When \textit{SIC1} expression was removed on glucose, both \textit{CDC14} containing plasmids were capable of rescuing the strain compared to the vector control [Figure 2.6, Glucose-uracil]. Therefore, the levels of CDK relative to Cdc14 phosphatase affect the ability of these cells to progress through the cell cycle.

Since the localization of Ace2 is controlled in a similar manner to Swi5, I investigated its localization when mitotic cyclins are sustained. The ratio of nuclear (defined by HTB2-mCherry) to cytoplasmic signal of Ace2-YFP was used to determine the timing and quantity of Ace2-YFP entry into the nucleus. Here, I report my findings on the small number of cells analyzed to date (n=21) [Figure 2.7].

In cells without locked mitotic cyclins (unpulsed), Ace2 entered the nucleus on average twelve minutes later than Swi5 entered relative to anaphase in previous
**Figure 2.6: Additional copies of CDC14 rescue CLB2kd lethality.** A diploid strain (\textit{cdc14/CDC14}-YFP \textit{CLB2,kd/CLB2 GAL-SIC1(2x) ura3/ura3}), was transformed with one of three \textit{URA3}-containing plasmids: vector control (pRS416), centromeric (pRS316-\textit{CDC14}), or integrating (pRS406-\textit{CDC14}). Transfomants were grown in galactose and plated in serial dilutions (10-fold) onto glucose or galactose that either lacked uracil or contained 5-FOA (which counterselects for the \textit{URA3} gene on the plasmids). While all strains were fully viable on Galactose-uracil (top left), the vector control was the only one unable to rescue on Glucose-uracil (when the \textit{SIC1} construct was turned off) (top right). This rescue is dependent on the plasmid since it does not persist on glucose + FOA for the centromeric plasmid (bottom right).
**Figure 2.7: Constitutive mitotic cyclin activity shows an effect on the initial entry timing and quantity of Ace2 nuclear localization.** In (A), the average timing in minutes from anaphase of the first nuclear entry of Ace2 is shown for unpulsed versus pulsed cells, divided into mothers and daughters. Error bars are standard deviation. Mothers and daughters showed no significant difference in timing in either case. In cells with locked mitotic cyclins (n=12), the average time before Ace2 nuclear entry increased, but the response was highly variable. Pulsed cells had an average mitotic cyclin level of 3x peak. The combined unpulsed (n=9) cells have an average entry time of 15 minutes, which is about 12 minutes after Swi5 entry (compare to Figure 2.2). (B) The amount of Ace2-YFP entering the nucleus (based on the nuclear to cytoplasmic ratio as in Figure 2.3) for unpulsed (red) daughters (filled) and mothers (no fill) was similar for both entries. The entry for pulsed cells (blue) was also similar for daughters (filled) and mothers (no fill). Overall, the amount of Ace2 localizing to the nucleus during the first entry was lower when mitotic cyclins were sustained.
Figure 2.7

A

Timing of first Ace2-YFP nuclear entry

B

Amount of Ace2-YFP nuclear entry
experiments [Figure 2.2A and Figure 2.4A], consistent with a previously published report of ten minutes (Sbia et al. 2008). The timing and amount of Ace2 entry did not vary appreciably between mothers and daughters [Figure 2.7]. While the duration of Ace2 nuclear localization was longer in daughters (average ± standard deviation (in minutes): 15 ± 4.2 versus 11 ± 3.0), the difference was not as substantial as previously reported (23 versus 7 minutes) (Di Talia et al. 2009). However, this can be attributed to the unusually large size of daughters at birth after the long metaphase arrest used in the protocol (Di Talia et al. 2009).

In cells with sustained mitotic cyclins (pulsed), Ace2 nuclear entry was affected, in a fashion distinct from that of Swi5. The timing of entry into the nucleus from anaphase was generally increased compared to cells without locked mitotic cyclins with a high variability in timing [Figure 2.7A]. This contrasts with the relatively unchanged initial entry of Swi5 [Figure 2.2A]. Additionally, the quantity of Ace2 in the nucleus during the first entry was generally lower in pulsed cells [Figure 2.7B]. These results are from only a few cells with a relatively high average mitotic cyclin level in pulsed cells (~3x peak) and therefore require further investigation. Still, the differences in regulation of the localization of these two transcription factors may prohibit Ace2 from contributing to mitotic exit progression when mitotic cyclins are sustained. However, the fact that Swi5 and Ace2 show different patterns of localization when mitotic cyclin is sustained suggests that CDK activity during the normal cell cycle affects them differently.
This could result from differences in localization (of the kinase, phosphatase, or transcription factors), affinities of the kinase and phosphatase for the transcription factors, or other mechanisms (see below)

**CONCLUSION**

Our results show that Swi5 translocates to the nucleus despite sustained mitotic cyclin levels with little difference in timing or amount of initial entry. This result is not surprising given the effect Swi5 has on the thresholds of mitotic cyclin for budding and cytokinesis. However, at first glance, it is inconsistent with the known CDK-dependent inhibition of Swi5’s nuclear localization (Moll *et al.* 1991).

Swi5 is a target of both CDK and Cdc14 phosphatase activity (Surana *et al.* 1991, Visintin R. *et al.* 1998) [Figure 2.5]. Studies from our laboratory show that Cdc14 is released from the nucleolus in a periodic fashion even when mitotic cyclins are sustained (Lu and Cross 2010). Therefore, we propose that the repeated Swi5 nuclear entries are a result of the oscillations in the kinase/phosphatase balance between CDK and Cdc14 activities [Figure 2.5]. In future experiments, I will determine the correlation between Cdc14 nucleolar release and Swi5 nuclear entry in individual cells.

Overall, these findings support the model we proposed for how cells progress through mitotic exit despite locked mitotic cyclins. In a normal cell cycle, the
relevant variable for ordering mitotic exit events may be the balance of competing kinase and phosphatase activities and their control over different events. Therefore, thresholds for particular events in mitotic exit may be set, in part, by the susceptibility of relevant targets to both the kinase and phosphatase activities. Preliminary analysis of the Swi5 homolog Ace2 showed that it may be incapable of entering the nucleus in the presence of sustained mitotic cyclin, demonstrating that even very similar proteins can respond differently to the kinase/phosphatase balance. Additionally, neither mitotic cyclin/CDK nor Cdc14 phosphatase is distributed uniformly in the cell (Bailly et al. 2003, Buttery et al. 2007, Hood et al. 2001, Pereira and Schiebel 2003, Pereira et al. 2002, Shou et al. 1999, Visintin R. et al. 1999, Yoshida et al. 2002). Therefore, local activity concentrations around substrates would be expected to play a role in setting the kinase/phosphatase balance. Accounting for the variables of phosphatase activity, substrate susceptibility, and local concentrations of kinase and phosphatase activities near substrates may explain how the rise and fall of CDK activity once per cell cycle maintains order and timing; however, these are not easily assayed at this time.
CHAPTER THREE
The contributions of selected Swi5 targets in promoting budding and cytokinesis.

INTRODUCTION

As transcription factors, Swi5 and Ace2 are responsible for a coordinated program of transcription during the transition from the end of mitosis into the subsequent cell cycle. Since we proposed that Swi5 plays a critical role in promoting budding and cytokinesis in the presence of locked mitotic cyclins, I sought to determine if particular Swi5 targets were critical for setting the mitotic cyclin inhibitory thresholds of mitotic events.

Swi5 was first described as an activator of the $HO$ endonuclease, responsible for mating type switching in yeast (Dohrmann et al. 1992, Nasmyth et al. 1987). In addition, Swi5 regulates the transcription of several genes involved in cell cycle progression, including an inhibitor of DNA replication ($CDC6$) (Piatti et al. 1995) and two cyclin family genes ($PCL2$ and $PCL9$) (Aerne et al. 1998, Tennyson et al. 1998). Together with Ace2, Swi5 is responsible for promoting transcription of the mitotic cyclin/CDK inhibitor $SIC1$ (Knapp et al. 1996, Toyn et al. 1997).

A more complete, unbiased list of Swi5 targets was recently generated by studies from our laboratory in collaboration with the laboratory of Bruce Futcher [Figure 3.1] (Di Talia et al. 2009). A wild type and a $swi5\Delta$ mutant strain were released
**Figure 3.1:** Swi5 targets identified previously by microarray (courtesy of Stefano Di Talia). Genes with differential expression between WT and the swi5Δ mutant are shown following release from a metaphase arrest. Interesting candidates discussed in this chapter are indicated with star (*). Null mutations of these targets are being tested to see if they have a similar effect on mitotic cyclin inhibitory thresholds as a swi5Δ mutant. As indicated by the color scale, yellow represents up-regulation while blue represents down-regulation compared to control (ratio of experiment/control). Black is a ratio close to one, and grey is missing data.
from a metaphase arrest, and samples were collected every 5 minutes. The resulting microarray data was aligned by the time of anaphase to increase accuracy, and hierarchical clustering was used to generate a list of genes whose expression during this period of the cell cycle is specifically activated by Swi5 [Figure 3.1].

From this list, targets were selected that might be responsible for the ability of Swi5 to promote budding and cytokinesis. Deletions of individual targets involved in shifting the thresholds might be expected have a similar effect to the SWI5 deletion [Figure 1.2B], intermediate effects if they are one of several contributors, or stronger effects if their transcription is decreased but not eliminated by a SWI5 deletion. Because of the specificity of Swi5’s effect on promoting budding and cytokinesis, I initially focused on proteins that were known to be involved in these processes and/or that localized to the bud neck. More details about the selected targets are discussed below.

RESULTS

In order to determine if an individual Swi5 target was involved in Swi5’s promotion of cytokinesis and budding, the timing of mitotic cyclin events was assayed using a strain with a deletion of the target gene in the presence of sustained mitotic cyclin (protocol in Figure Intro-1). The cells were followed after release by timelapse microscopy [as in Figure 1.4]. For individual cells, the
amount of fluorescently labeled mitotic cyclin was compared to the timing of events in mitotic exit (cytokinesis initiation, completion and rebudding). For statistical comparisons, the data were binned to ensure a sufficient number of cells within a given range of mitotic cyclin levels. Because some events were not observed for some cells during the time of the experiment (360 minutes) and could not be accurately assigned a number value, a Mann-Whitney test was performed comparing the rank order of the values within a bin.

The targets chosen for analysis were involved in cytokinesis and budding or localized to the bud neck. They were also among those with the strongest dependence on Swi5 and Ace2 for their activation: \textit{YPL158c, NIS1, and CYK3} (Di Talia \textit{et al.} 2009). In cells without mitotic cyclin sustained, deletions in these genes did not cause delays in the events assayed when compared to wild type cells (data not shown); however, as with \textit{swi5Δ} mutants, the sensitized background of sustained mitotic cyclins may reveal a synthetic relationship in promoting the events of cytokinesis and bud formation.

\textit{YPL158c (AIM44)} was previously identified as a Swi5 target (Doolin \textit{et al.} 2001) and localizes to the bud neck (Huh \textit{et al.} 2003). However, the function of Aim44 is undetermined. A yeast two hybrid screen showed that it binds to the SH3 domains of four proteins that are also localized to the bud neck: Cyk3, Hof1, Boi1, and Boi2 (Tonikian \textit{et al.} 2009). In addition, a deletion in \textit{AIM44} was
identified by two independent high-throughput screens as synthetically lethal in conjunction with a deletion in \textit{SLT2} (Costanzo \textit{et al.} 2010, Tong \textit{et al.} 2004), a mitogen-activated protein kinase (MAPK) that is active during times of polarized growth (Madden \textit{et al.} 1997, Zarzov \textit{et al.} 1996).

When compared to wild type, \textit{aim44}Δ mutants significantly shifted the thresholds for all mitotic exit events assayed [Figure 3.2]. Therefore, Aim44 promotes budding and cytokinesis in cells where mitotic exit is delayed by sustained mitotic cyclins; however, the effect was less than that seen in \textit{swi5}Δ mutants. This suggests Swi5 promotes cytokinesis and budding by regulating transcription of \textit{AIM44} and that of additional genes.

The second target of interest, Nis1, localizes to the bud neck during G2 and mitosis (Iwase and Toh-e 2001). Nis1 physically interacts with septins, which form a ring beneath the plasma membrane and act as an essential scaffold for the recruitment of the cytokinetic machinery (Iwase and Toh-e 2001, McMurray and Thorner 2009). Nis1 also physically interacts with several components of the mitotic signaling network, including kinases Gin4 and Kcc4 (Iwase and Toh-e 2001). This pathway inhibits the ability of Swe1 kinase to inactivate mitotic cyclin/CDK complexes, which then promote isotropic growth in the emerging bud; in the event of delayed bud emergence, Swe1 inhibits mitotic cyclin/CDK
Figure 3.2: **Aim44 promotes budding and cytokinesis when mitotic cyclin is sustained.** Data from timelapse movies shown in scatter-plots with mitotic cyclin pulse concentration (as a fraction of peak) versus time (in minutes) for individual cells for cytokinesis initiation (top), completion (center), or rebudding (bottom). *aim44Δ (ypl158cΔ)* (purple squares) plotted with wild type (blue circles) and *swi5Δ* (red triangles) controls in background. Dashed line represents the end of the movie at six hours; points above this line are therefore cells that did not accomplish the event before the end of the movie. Results from a Mann-Whitney test between the *aim44* and wild type strains are indicated by boxes (approximating bin sizes horizontally) above the graphs (*p*-value given and represented by shade, with color denoting which of the two strains has the longer median time in a given bin).
Figure 3.2

![Graphs showing cytokinesis initiation, completion, and rebudding times with p-values for WT, swi5Δ, and aim44Δ strains.](Image)
complexes and delays cell cycle progression (Keaton and Lew 2006, Sreenivasan and Kellogg 1999). While the function of Nis1 has proved elusive, its interactions implicate a role in cytokinesis and budding.

When *NIS1* was deleted, the effect on the mitotic cyclin inhibitory thresholds was subtle but still constituted a statistically significant difference compared to wild type for cytokinesis initiation and completion [Figure 3.3]. This is with the caveat that the number of *nis1Δ* cells assayed with mitotic cyclin levels significantly higher or lower than the peak (represented by the left and rightmost bins in Figure 3.3) is presently low, and apparent significance is reliant on bin size. *nis1Δ* mutant had no significant effect on the ability of cells to rebud. With additional data, this result may implicate Nis1 as an additional target contributing specifically to Swi5’s ability to promote cytokinesis.

The final target tested, Cyk3, also localizes to the bud neck during cytokinesis and activates the chitin synthase to promote subsequent septum formation, contributing to the actomyosin-independent cytokinesis pathway (Jendretzki et al. 2009, Korinek et al. 2000, Nishihama et al. 2009).

When *CYK3* was deleted, the effect on all thresholds was intermediate, strongest for budding, but not significant overall [Figure 3.4]. Therefore, Cyk3 may be a
Figure 3.3: Nis1 specifically promotes cytokinesis when mitotic cyclin is sustained. Data from timelapse movies shown in scatter-plots with mitotic cyclin pulse concentration (as a fraction of peak) versus time (in minutes) for individual cells for cytokinesis initiation (top), completion (center), or rebudding (bottom). $nis1\Delta$ (orange squares) plotted with wild type (blue circles) and $swi5\Delta$ (red triangles) controls in background. Dashed line represents the end of the movie at six hours; points above this line are therefore cells that did not accomplish the event before the end of the movie. Results from a Mann-Whitney test between the $nis1\Delta$ and wild type strains are indicated by boxes (approximating bin sizes horizontally) above the graphs ($p$-value given and represented by shade, with color denoting which of the two strains has the longer median time in a given bin).
Figure 3.3
Figure 3.4: Cyk3 may promote budding when mitotic cyclin is sustained. Data from timelapse movies shown in scatter-plots with mitotic cyclin pulse concentration (as a fraction of peak) versus time (in minutes) for individual cells for cytokinesis initiation (top), completion (center), or rebudding (bottom). cyk3Δ (green squares) plotted with wild type (blue circles) and swi5Δ (red triangles) controls in background. Dashed line represents the end of the movie at six hours; points above this line are therefore cells that did not accomplish the event before the end of the movie. Results from a Mann-Whitney test between the cyk3Δ and wild type strains are indicated by boxes (approximating bin sizes horizontally) above the graphs (p-value given and represented by shade, with color denoting which of the two strains has the longer median time in a given bin).
Swi5 target partially responsible for promoting budding despite locked mitotic cyclins. Interestingly, a recent study showed that overexpression of mitotic cyclin/CDK activity inhibits the localization of Cyk3 to the bud neck (Meitinger et al. 2010). The localization of Cyk3 in cells with sustained mitotic cyclin could vary from that caused by overexpression and needs to be determined to understand any interpretation of these results.

CONCLUSION

Of the Swi5 targets assayed, none was individually able to replicate the effect seen in the swi5Δ strain [Figure 1.4]. Aim44 contributed the most robustly to all three events assayed [Figure 3.2], and its location at the bud neck could allow it to affect both budding and cytokinesis. However, little is known about its direct action there. Nis1 contributed modestly but significantly to promoting cytokinesis, and it is known to physically interact with the cytokinetic machinery [Figure 3.3]. Cyk3, in contrast, had its strongest effect in promoting budding, but this result was not robustly significant [Figure 3.4]. Some of these conclusions require additional data collection. Furthermore, the effect of deleting Swi5 and sustaining mitotic cyclins on the expression level of these targets will be investigated by isolating RNA during the release and performing RTqPCR.

The intermediate effects seen from the preliminary analysis so far could be the result of a coordinated process controlled during an important transition in the cell
cycle. As a major transcription factor activated during mitotic exit, Swi5 is responsible for the transcription of a variety of targets that regulate the concomitant events. Therefore, deletion of an individual target may never be able to phenocopy the effect of swi5Δ deletion, which results in the simultaneous decrease in expression of all Swi5 targets.

Our previous criteria of bud neck localization and direct involvement in the processes of budding and cytokinesis unintentionally biased the selection for Swi5 targets that have relatively narrow effects. Because the target responsible for Swi5’s ability to promote cytokinesis and rebudding may be one with more pleiotropic roles, it is interesting that Swi5 is responsible for the transcription of two homologous cyclin family genes, PCL2 and PCL9 (Aerne et al. 1998, Di Talia et al. 2009, Tennyson et al. 1998). Both are responsible for binding to and activating the non-essential CDK Pho85 (Measday et al. 1997). In the future, it will be interesting to see if these cyclins could contribute to the effect of Swi5 on cytokinesis and budding. Pho85 CDK activity has been implicated in many events of the cell cycle during mitotic exit and G1, including the efficient restoration of growth after cell cycle delays when Cdc28 CDK activity is inhibited (Lee J. et al. 1998, Measday et al. 1997, Wysocki et al. 2006). Consequently, (Pcl2 and Pcl9-dependent) Pho85 CDK activity could be the effector predominantly responsible for the bulk of Swi5’s promotion of cytokinesis and rebudding during the delay caused by sustained mitotic cyclin.
Because Pho85 is activated by the binding of ten different cyclins, a \textit{PHO85} deletion would remove additional activities not known to be affected by the deletion of \textit{SWI5} (Huang \textit{et al.} 2007). Additionally, \textit{PHO85} deletion is known to hyperactivate Swi5 (Measday \textit{et al.} 2000). Therefore, a double deletion of the two cyclins (\textit{pcl2 pcl9}) whose expression is Swi5-dependent will allow us to specifically assay the possibility that Swi5 promotes budding and cytokinesis in the presence of locked mitotic cyclins by activating Pho85-CDK activity.
CHAPTER FOUR

Identifying novel Cdc14 phosphatase targets and the physiological relevance of their dephosphorylation.

INTRODUCTION

The focus of the preceding chapters has been on the regulation by CDK of events of mitotic exit; however, the counteracting phosphatase Cdc14 is also important for understanding this regulation. As discussed in Chapter Two, the balance of CDK and Cdc14 activities may be the relevant variable in ordering mitotic events. Therefore, I investigated novel Cdc14 substrates involved in mitosis and cytokinesis identified by a postdoctoral fellow in my laboratory, Joanna Bloom, in collaboration with Ileana Cristea in the laboratory of Brian Chait.

Cdc14 is essential for mitosis and promotes inactivation of CDK by dephosphorylating three key substrates: (1) Cdh1, which activates the APC^{Cdh1}, (2) Swi5, a transcription factor which upregulates transcription of the CDK inhibitor SIC1, and (3) Sic1 itself, preventing its ubiquitin-dependent degradation (Jaspersen et al. 1998, Schwab et al. 1997, Visintin R. et al. 1998). While the role of Cdc14 in coordinating the decrease in CDK activity has been well characterized, less is known about its other targets in mitosis; it is known, however, that CDK and Cdc14 share a preference for phospo-Ser/Thr-Pro motifs.
Therefore, identifying mutual targets will give us a better understanding of how these competing activities regulate mitotic events.

Cdc14 phosphatase dephosphorylates substrates in several mitotic processes. These substrates include Sli15, Ask1, Fin1, and Ase1, which are critical for stabilizing the anaphase spindle (Higuchi and Uhlmann 2005, Khmelinskii et al. 2007, Pereira and Schiebel 2003, Woodbury and Morgan 2007). A role for Cdc14 in cytokinesis has also been implied. Cdc14 localizes to the bud neck when released (Bembenek et al. 2005), and in cells where CDK activity is ectopically inactivated without Cdc14 release from the nucleolus, the actomyosin ring shows defects in contraction and cell separation (Corbett et al. 2006, Hwa Lim et al. 2003, Yeong 2005, Yeong et al. 2002). However, the substrates responsible for Cdc14’s promotion of cytokinesis are not known.

Cdc14 phosphatase activity is restricted to mitotic exit due to its subcellular localization. The nucleolar protein, Net1, sequesters Cdc14 in the nucleolus until anaphase (Shou et al. 1999, Traverso et al. 2001, Visintin R. et al. 1999). Two signaling pathways converge at the end of the cell cycle to release Cdc14: the Cdc14 early anaphase release (FEAR) pathway and the Mitotic Exit Network (MEN). During early anaphase, FEAR triggers a qualitatively minor release of Cdc14, which remains nuclear, accumulates at SPBs, and is not sufficient for mitotic exit (Shou et al. 1999, Stegmeier et al. 2002, Visintin R. et al. 1999,
Visintin R. et al. 1998). When the daughter-bound SPB reaches the bud, the MEN signaling pathway is activated and fully releases Cdc14 (Bardin et al. 2000), connecting proper spindle orientation and nuclear segregation with progression through mitosis.

Previous studies using immunopurification and mass spectrometry (MS) analysis of Cdc14 in asynchronous cells identified a known Cdc14 substrate (Sli15) and novel cell-cycle proteins (Cristea et al. 2005). However in asynchronous cells, Cdc14 would be primarily restricted to the nucleolus. Therefore, Joanna Bloom and Ileana Cristea repeated such immunopurifications in a strain with the nucleolar anchor NET1 deleted (net1Δ). In these cells, Cdc14 was released throughout the cell cycle and therefore more likely to interact with substrates. For comparison, a conditional mutant in the MEN pathway (cdc15-2) was used at the restrictive condition, when Cdc14 would be primarily sequestered. Cdc14 was immunopurified from net1Δ and cdc15-2 strains, and the associated proteins were analyzed by MS [Figure 4.1; Joanna Bloom and Ileana Cristea]. The released Cdc14 in the net1Δ strain interacted with more proteins than sequestered Cdc14 in the cdc15-2 strain; however, both conditions identified potentially interesting targets. The interactions seen in predominately-sequestered conditions may have occurred post-cell lysis.
Figure 4.1: Identification of proteins associated with released versus sequestered Cdc14 (courtesy of Joanna Bloom). Left, Cdc14-5xGFP, released in *net1Δ* cells or sequestered in *cdc15-2* cells, was immunopurified with a polyclonal GFP antibody conjugated to magnetic beads. Eluates were resolved on SDS-PAGE gels and stained with Coomassie blue. Right, Cdc14-associated proteins identified from the gel, shown on left, classified into functional groups. Highlighted are two functionally redundant formins: Bni1 (blue) and Bnr1 (red). Stars indicate proteins previously identified as either CDK substrates (*) (Ubersax *et al.* 2003) or preferred substrates of Clb5/CDK compared to Clb2/CDK (**)(Loog and Morgan 2005).

Importantly, many of these Cdc14 interacting proteins are also predicted to be CDK targets [Figure 4.1] (Ubersax et al. 2003).

Because the interactions between a phosphatase and its targets may be too transient to detect by these methods, many potentially interesting Cdc14 interactions may have been missed. However, Mutations in the active site of protein tyrosine phosphatases (PTPs) stabilize interactions between the phosphatase and its substrates (“substrate-trapping” mutants) (Flint et al. 1997, Jia et al. 1995), and structurally, human Cdc14B is very similar to PTP-1B, including alignment of the Cys and Asp residues in their active sites (Gray et al. 2003). Additionally, these residues are conserved in budding yeast Cdc14, and their mutation leads to the inactivation of Cdc14 (Taylor et al. 1997, Wang et al. 2004).

Based on these results, our lab generated mutants in these residues (cdc14C283S and cdc14D253A) [Figure 4.2A; Joanna Bloom]. In vitro, these
Figure 4.2: Enhanced binding of Cdc14 interactors to “substrate trapping” CDC14 mutants in vitro (courtesy of Joanna Bloom). (A) Sepharose beads were coated with similar amounts of recombinant GST alone, GST tagged wild type Cdc14 (GST-WT) or GST tagged catalytically inactive Cdc14 mutants (GST-C283S, GST-D253A). Eluates were resolved on SDS-PAGE gels and stained with Coomassie blue. (B) Beads in (A) were incubated with extract from yeast strains with the indicated PrA-tagged gene. Affinity purified proteins were resolved on SDS-PAGE gels and immunoblotted with a rabbit IgG antibody. (C) Quantification of binding of Bni1-PrA, Bnr1-PrA, Kar9-PrA and Sli15-PrA to GST-C283A and GST-D253A over binding to GST-WT. Quantification represents three independent experiments.
mutants were used in an immunopurification from strains with different associated proteins from Figure 4.1 tagged with Protein A (PrA) [Figure 4.2B; Joanna Bloom]. The mutants showed increased binding to the known Cdc14 target (Sli15) and to several of the novel, associated proteins (Bni1 and Bnr1; also Kar9, not shown in A) [Figure 4.2C; Joanna Bloom].

Our lab compared the binding of prospective Cdc14 substrates with either Cdc14 or the substrate-trapping mutant (cdc14D253A) in vivo [Figure 4.3; Joanna Bloom]. Co-immunoprecipitations after transient expression of Cdc14 (wild type or mutant) showed that known Cdc14 target Sli15 and novel potential substrates (Bni1 and Bnr1) showed enhanced binding to the substrate-trapping mutant. Net1, the nucleolar anchor, bound less to the substrate-trapping mutant, possibly because the tighter binding to other targets decreased the pool available to bind Net1.

Our lab confirmed that Cdc14 not only bound but also dephosphorylated these novel targets in vivo and in vitro. In cells overexpressing Cdc14 or the catalytically inactive, substrate-trapping mutant, the mobility of both Sli15 and the formins Bni1 and Bnr1 shifted in the presence of wild type but not mutant Cdc14 [Figure 4.4A; Joanna Bloom]. In addition, an in vitro phosphatase assay was
Figure 4.3: Enhanced binding of Cdc14 interactors to substrate trapping CDC14 mutants \textit{in vivo} (courtesy of Joanna Bloom). Immunopurifications of FLAG-tagged Cdc14 from strains transiently expressing wild-type Cdc14 (\textit{GALS-WT-FLAG}) or the substrate-trapping Cdc14 mutant (\textit{GALS-D253A-FLAG}) in combination with the specified 6HA-tagged proteins (upper panels) and whole cell extract from these strains (lower panels). Immunoprecipitates and extracts were resolved on SDS-PAGE gels and immunoblotted with an antibody to the HA tag or to Cdc14.
Figure 4.4: Confirmation of new Cdc14 substrates in vivo and in vitro (courtesy of Joanna Bloom). (A) Immunoblot of the indicated 6HA-tagged proteins in the absence of overexpressed Cdc14 (-) or following transient expression of wild-type Cdc14 (GALS-WT) or the “substrate-trapping” Cdc14 mutant (GALS-D253A). The asterisk indicates a Bni1-6HA degradation product. (B) In vitro phosphatase assay of Bni1 and Sli15. The 6HA-tagged substrates were immunopurified from cells transiently overexpressing Clb2 to ensure their phosphorylation. Purified proteins were then incubated with buffer (untreated), recombinant GST alone (GST), GST-tagged wild-type Cdc14 (GST-WT), GST-tagged catalytically inactive Cdc14 mutants (GST-C283S and GST-D253A), or λ phosphatase. Immunoprecipitates were analyzed by immunoblotting with an antibody to HA. (C) In vitro phosphatase assay after immunopurification of 6HA-tagged Bni1 following transient expression of GAL-CLB2 as in (B). Immunoprecipitates were analyzed by immunoblotting with an antibody to phospho-serine/phospho-threonine residues or an antibody to HA.
performed with substrates isolated from cells overexpressing the mitotic cyclin
*CLB2* [Figure 4.4B,C; Joanna Bloom]. When exposed to wild type Cdc14, Bni1
and Sli15 were both dephosphorylated similar to λ phosphatase treatment, as
evidenced by a mobility shift on SDS-PAGE, but not when exposed to the
catalytically inactive, substrate-trapping mutant [Figure 4.4B]. Similarly, when
probed with an antibody that recognizes phospho-serine and phospho-threonine
residues, Bni1 phosphorylation was decreased after incubation with wild type but
not mutant Cdc14 [Figure 4.4C; Joanna Bloom]. While results for Bnr1 were
similar, we are slightly less confident in them due to the smaller mobility shift
[Figure 4.4A; Joanna Bloom] and less reproducible results from the anti-phospho-
serine/threonine antibody (data not shown).

In summary, biochemistry experiments conducted by Joanna Bloom in our lab in
collaboration with the laboratory of Brian Chait have identified several new Cdc14
targets using immunopurification with MS analysis and a Cdc14 mutant designed
to distinguish between substrates and other associated proteins. These new
substrates include the formins, Bnr1 and Bni1, and several other proteins
involved in mitotic processes. Therefore, to better understand the role of Cdc14
in cytokinesis, I investigated the physiological consequences of Bni1 and Bnr1
dephosphorylation by Cd14 phosphatase.
Formins are a conserved family of actin-nucleating proteins involved in many actin-dependent processes, including the contraction of the actomyosin ring during cytokinesis (Evangelista et al. 2002). The defining feature of formins is adjacent formin homology domains, FH1 and FH2 (Goode and Eck 2007, Higgs and Peterson 2005). The proline-rich FH1 domain recruits profilin-actin complexes to the elongating actin filament while the FH2 domain is responsible for nucleating and capping the filament. Bni1 and Bnr1 are the only formins in yeast and have partial functional redundancy (Imamura et al. 1997). Individual deletions of these formins are viable; however, in the absence of both formins, cells fail to form an actomyosin ring (Tolliday et al. 2002). Phosphorylation of Bni1 does not affect actin nucleation in vitro (Moseley and Goode 2005), so Cdc14-dependent dephosphorylation is unlikely to affect formin activity.

Of interest, both Bni1 and Bnr1 are localized to the bud neck, but during different periods of the cell cycle. Bnr1 arrives during emergence of the bud and leaves just before actomyosin ring contraction (Buttery et al. 2007, Gladfelter et al. 2001, Kamei et al. 1998). By contrast, Bni1 is localized to sites of growth including the bud tip and arrives at the bud neck after the mitotic spindle is disassembled, just prior to cytokinesis (Buttery et al. 2007, Ozaki-Kuroda et al. 2001). Deletion of BNI1 causes delays and occasional failures in actomyosin ring contraction (Vallen et al. 2000). Conversely, overexpression of BNR1 causes a cytokinesis
defect (Kikyo et al. 1999). These results could be explained by a requirement to switch formins at the bud neck during anaphase for efficient cytokinesis.

Since this alteration of formin localization at the bud neck occurs during anaphase when Cdc14 is released from the nucleolus, we hypothesized that the dephosphorylation of the formins could cause of the switch. I therefore set out to test if the switch in localization of the formins during anaphase results from Cdc14 dephosphorylation.

RESULTS

I first confirmed the timing of formin localization during a synchronized cell cycle [Figure 4.5]. Cells with Bni1-GFP or Bni-GFP were released from a metaphase block and visualized. Bnr1-GFP showed bud neck localization starting at the time of the block and decreasing 20 minutes after release. Bni1-GFP by contrast was not present at bud necks during the metaphase arrest, but arrived around the time Bnr1 left. The switch between formins at the bud neck was coincident, and strikingly, this timing was similar to the time at which Cdc14 is released from the nucleolus in the same protocol (Ying Lu, personal communication). Additionally, mutants in Cdc14 (cdc14-1) or in the signaling pathways leading to its release (cdc15-2) show Bnr1 localized to the bud neck and Bni1 absent from the bud neck (data not shown), suggesting that Cdc14 plays a role in formin localization. Together with the biochemical evidence that
Figure 4.5: The timing of the switch in formin localization is coincident with Cdc14 release. (A) After a metaphase arrest (M), cells were released synchronously, and the localization of the formins (Bni1 or Bnr1) at the bud neck was scored. The timing of Bnr1 leaving the bud neck is coincident with Bni1 arriving. This switch is also coincident with the timing of Cdc14 release from the nucleolus during the same protocol, grey box (Ying Lu, personal communication). (B) Representative images from the experiment in (A). Scale bar is 10μm.
Figure 4.5

A

Percent Bud Neck Localization

![Graph showing percent bud neck localization over time after release](image)

- Bni1-GFP
- Bnr1-GFP
- Estimated time of Cdc14 release

B

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</table>
Cdc14 dephosphorylates the formins, these results are consistent with the hypothesis that Cdc14 dephosphorylation is directly responsible for formin localization. Subsequent studies were undertaken to verify this hypothesis.

Mutations within the nuclear export sequence (NES) of Cdc14 were reported to allow destruction of mitotic cyclins but block cytokinesis (Bembenek et al. 2005). Using this conditional, separation-of-function mutant, I investigated the effect of Cdc14 release when it is incapable of reaching cytoplasmic targets. At the restrictive temperature, \textit{cdc14}\textsuperscript{ΔNES} did cause cells to arrest with large buds and replicated DNA (data not shown). However in my hands, Clb2 levels were not degraded with normal efficiency during the \textit{cdc14}\textsuperscript{ΔNES} arrest [Figure 4.6A]. This discrepancy negates the use of the \textit{cdc14}\textsuperscript{ΔNES} as a separation-of-function mutant. Since the mutant cdc14\textsuperscript{ΔNES} protein was not visible at the bud neck when compared to wild type (data not shown) as reported (Bembenek et al. 2005), localization of the formins during the cdc14\textsuperscript{ΔNES} arrest was still assayed. I observed that Bnr1 localized to the bud neck of large budded cells [Figure 4.6B]; however, given that Clb2 levels remain high in these cells, this does not add anything significant to previous findings with other Cdc14 mutants (see above).

I next determined the localization of formins in cells maintained in metaphase by depletion of Cdc20 and induced to overexpress Cdc14 [Figure 4.7]. An empty
Figure 4.6: A *cdc14ΔNES* strain does not degrade Clb2 during arrest. (A)
Protein levels of mitotic cyclin Clb2 are shown for an asynchronous culture (A), at a G1 arrest (α), or after release (in minutes) to 37°C. Alpha factor was added at 60 min after release. The *cdc14ΔNES-GFP* mutant did not result in the degradation of Clb2 as reported by Bembenek et al, 2005. (B) Cells with BNR1-GFP and an untagged version of the *cdc14ΔNES* mutant arrested with Bnr1 present at the bud neck of most large budded cells (180 min after release). Scale bar is 10μm.
Figure 4.6

A

CDC14-GFP

αCib2

αPgk1

cdc14ΔNES-GFP

αCib2

αPgk1

B

Bnr1-GFP

Bar scale
Figure 4.7: Localization of formins during Cdc14 overexpression at a metaphase arrest. Strains were arrested in metaphase and then galactose was added to induce the expression of CDC14 from the plasmids (vector control, wild type CDC14, or the catalytically inactive cdc14D253A mutant). Presence of the formin at the bud neck is shown during arrest (M) and at one and two hours after addition of galactose (1, 2). While the result shows CDC14 overexpression caused a relocalization of both formins, further examination showed that the overexpression was also causing exit from mitosis (assayed by actomyosin ring breakdown and DNA content; data not shown).
vector or plasmids expressing wild type or a catalytically inactive mutant of Cdc14 were transformed into strains with a fluorescently tagged formin. A truncated galactose inducible promoter (\textit{GALL}) was used to lessen the overexpression (Mumberg \textit{et al.} 1994). When wild type \textit{CDC14} was overexpressed, I observed a change in both of the formins’ localizations at the bud neck after two hours [Figure 4.7]; however, cells expressing wild type Cdc14 were also no longer arrested in metaphase and instead exited mitosis as assayed by breakdown of their actomyosin rings and DNA replication (data not shown). This experiment was repeated in a strain with \textit{CDH1} deleted to maintain high levels of mitotic cyclin, but the same release from the block was observed (data not shown). Therefore, the change in localization of the formins seen could not be directly attributed to Cdc14 and may be explained by the normal pattern of bud neck localization during cell cycle progression.

Since overexpression of Cdc14 during a metaphase arrest was unfeasible, I instead sought a system where an endogenous level of Cdc14 could be released from the nucleolus without cell cycle progression. This was accomplished through the overexpression of separase (\textit{GALS-ESP1}) in cells with Cdh1 and Cdc20 inactivated, causing continuous Cdc14 release during a prolonged anaphase [Figure 4.8A] (Lu and Cross 2009). In a normal cell cycle, separase (Esp1) is kept inactivated by a chaperone and inhibitor called securin (Pds1) (Shirayama \textit{et al.} 1999). When the APC^{Cdc20} becomes activated and degrades...
Figure 4.8: Cells sustained in anaphase with Cdc14 release show a coincident relocalization of the formin Bnr1.  

(A) Previous experiments (Lu and Cross, 2009) show that when cells lacking CDH1 are arrested in metaphase (by depletion of Cdc20) and released by the expression of separase (ESP1) to anaphase, Cdc14 is released for a sustained period before cell cycle progression.  Left, Quantitative measurement of Cdc14 release during the experiment.  The histogram of r values ($r = [\text{Cdc14max} - \text{Cdc14min}] / [\text{Net1max} - \text{Net1min}]$ when the max and min values are the average intensity values of the maximum or minimum 5% pixels within a cell) shift to the left when Cdc14-YFP is more dispersed than its nucleolar anchor Net1-CFP.  This occurs between 1.5 hours after ESP1 expression was induced.  Frequency of cells in metaphase (red line), anaphase (yellow line), and after rebudding (green line) is represented.  Cells are unable to progress out of anaphase for 3-4 hours after induction.  Right, Clb2 cyclin levels are maintained throughout the experiment.  

(B) MET-CDC20 cdh1 GAL-ESP1 BNR1-GFP strain was arrested and either induced with ESP1 (+, in green) as in (A) or not (−, blue).  The localization of Bnr1-GFP to the bud neck is graphed.  The percent of large budded cells was used to show cell cycle progression and is shown by the line graph.
Figure 4.8

**A**

*cdh1Δ GALS-ESP1, cdc20Δ + Gal*

- **0 hrs**
- **0.5 hrs**
- **1 hrs**
- **1.5 hrs**
- **2 hrs**
- **2.5 hrs**
- **3 hrs**
- **4 hrs**

**Cdc14 release statistics (r)**

- **Metaphase**
- **Anaphase**
- **Rebudded**

**B**

- **- ESP1 + ESP1**

**Bnr1 localization to bud neck:**
- **Large budded:**

Lu and Cross (2009)
Pds1 (Yeong et al. 2000), Esp1 cleaves cohesins, allowing sister chromatid separation (Uhlmann et al. 2000). In the procedure I used, \textit{ESP1} is overexpressed during a metaphase arrest caused by depletion of Cdc20 in a strain with \textit{CDH1} deleted. Without the activators of the APC (Cdc20 and Cdh1), mitotic cyclin degradation is completely inhibited [Figure 4.8A] (Schwab \textit{et al.} 1997, Wasch and Cross 2002). The overexpression of Esp1, however, induces anaphase (after 1 hr \textit{ESP1} expression) and Cdc14 release (after 1.5 hrs \textit{ESP1} expression) but cell cycle progression is significantly delayed [Figure 4.8A] (Lu and Cross 2009, Sullivan and Uhlmann 2003). The prolonged release of Cdc14 is most likely due to the absence of Cdh1, which normally contributes to resequestration (Visintin C. \textit{et al.} 2008).

I employed this assay to determine if endogenous Cdc14 release without cell cycle progression was sufficient to shift the localization of the formins. Cells were either maintained in a metaphase arrest (-ESP1) or released to anaphase by induction of GALS-ESP1 (+ESP1) [Figure 4.8B]. Consistent with our hypothesis that Cdc14 affects formin localization, the formin Bnr1 left the bud neck at a time coincident with the release of Cdc14. Cells remained large budded during the timecourse, indicating that cell cycle progression was delayed.

Further investigation is necessary to substantiate this result. One caveat, for example, is that the current control is not optimal since control cells remain
arrested in metaphase. In future experiments, a control that advances into anaphase without releasing Cdc14 will be used for a more appropriate comparison (using \textit{GAL1-TEV1/SCC1-TEV}, in which inducible Tobacco etch virus protease cleaves the Scc1 cohesin subunit at an engineered site) (Sullivan and Uhlmann 2003, Uhlmann \textit{et al.} 2000). The localization of Bni1 during this protocol has yet to be assayed. Still, this result implicates Cdc14 dephosphorylation as the trigger for the exchange in formin localization seen during anaphase and gives physiological importance to the biochemical observation that Bni1 and Bnr1 are Cdc14 phosphatase substrates.

CONCLUSION

Novel proteins that interact biochemically with the essential Cdc14 phosphatase were successfully identified and shown to preferentially bind a substrate-trapping mutant (Joanna Bloom and Ileana Cristea). Furthermore, these proteins are dephosphorylated \textit{in vivo} and \textit{in vitro} by Cdc14 (Joanna Bloom). I focused subsequent studies on the formins, which nucleate actin cables to build and contract the cytokinetic ring. While the timing of a switch in formin localization at the bud neck is coincident with Cdc14 nucleolar release [Figure 4.5], a direct effect has been more difficult to show. However, arresting cells in the presence of a long endogenous release of Cdc14, I observed that localization of Bnr1 is altered by Cdc14 release [Figure 4.8B].
In the future, I plan to look at the ability of the formins to bind to the substrate-trapping mutant during a synchronized release from metaphase [timecourse as in Figure 4.5; pullout as in Figure 4.2]. Binding of the formins to the mutant Cdc14 is expected to decrease, coincident with Cdc14 release in the cells, due to the assumed dependency of substrate trapping mutant interactions on the phosphorylation of substrates.

Further studies could look into the physiological relevance of other targets identified in the biochemical experiments (data not shown). In addition to the formins, novel Cdc14 substrates identified are involved in spindle orientation, SPB duplication, and bud formation. Overall, identifying Cdc14 targets involved in mitotic exit events is essential to understanding how a cell that has accumulated CDK-dependent phosphorylations throughout the cell cycle returns to a naïve G1 state.
DISCUSSION

The exit from mitosis is an important transition during the cell cycle. The CDK that has driven many of the events is inhibited, and Cdc14 phosphatase is activated to dephosphorylate many mutual targets. All this serves to reset the cell cycle control system, allowing progeny cells to enter G1 in a naïve state, sensitive to CDK regulation again.

Peak mitotic cyclin permits mitotic exit

In studies led by Benjamin Drapkin, we tested the effect of sustaining mitotic cyclins during mitotic exit. Using an assay that allowed metaphase-arrested cells to be loaded with stable mitotic cyclin, single cells were assayed for their ability to complete mitotic exit events including spindle disassembly, cytokinesis, and budding in the subsequent cell cycle (Drapkin et al. 2009). The amount of stable cyclin produced was measurable in individual cells and was compared to the peak reached during a normal cell cycle.

From these studies, we concluded that peak mitotic cyclin levels seen in a normal cell cycle do not prevent cells from exiting mitosis. Instead, events are delayed by sustained mitotic cyclin in a dose-dependent manner. Based on these findings, a simple ratchet model of CDK activity is insufficient to explain the ordering of mitotic entry and exit.
The role of Swi5 in promoting cytokinesis and rebudding

To further understand the inhibitory thresholds of mitotic cyclin in the events of mitotic exit, I investigated the role of a key regulator during this cell cycle transition. Swi5, along with its homolog Ace2, activates the transcription of a regulon of genes during the transition from mitotic exit to G1. When *SWI5* was deleted in cells, the delays in mitotic exit events caused by sustaining mitotic cyclins were increased specifically for cytokinesis and budding [Figure 1.2]. The specificity of Swi5’s effect for these two processes and the strict relationship between protein level and kinase activity indicated that it was not likely due to an effect on CDK activity (Drapkin *et al.* 2009). Therefore, in wild type cells with mitotic cyclin sustained, Swi5 promoted cytokinesis and rebudding.

The synthetic relationship between sustaining mitotic cyclin and eliminating *SWI5* allows us to investigate the regulation of these proteins in a normal cell cycle. *swi5Δ* had only minor effects on the timing of cytokinesis and no discernible effect on rebudding [Figure 1.1]. However, by sustaining mitotic cyclins, we have created a sensitized background that may amplify the effect of genes like Swi5, which otherwise have subtle phenotypes or redundant pathways. Furthermore, our assay lends itself to a quantitative description of more subtle contributions than traditional all-or-none genetics (including delays and dosage sensitivity), which is a distinct advantage when investigating pathways with likely
redundancies. Additionally, Swi5's ability to promote cytokinesis and rebudding may be important during recovery from a spindle checkpoint arrest when mitotic cyclin levels accumulate to levels above the peak of the normal cell cycle (Drapkin et al. 2009, Hwang et al. 1998). Swi5 exclusively affected the ability of cells to complete cytokinesis and form a new bud; testing for similar synthetic relationships of sustained mitotic cyclin with other mitotic regulators may increase our understanding the rate limiting steps involved in other events, including spindle disassembly.

The minor effects of a swi5Δ mutant in a normal cell cycle could be due to redundancy with the remaining homologous transcription factor, Ace2, or other unknown factors. To further investigate the contributions of these two transcription factors, we would extend our current studies to look at an ACE2 deletion and the double mutant ace2Δ swi5Δ. While Swi5 and Ace2 have known differences in regulation and targets, their significant overlap may be sufficient to mask effects of individual deletions. Laboratory strains and growth conditions may artificially downplay the importance of this redundancy. For example in pathogenic yeast with only one orthologue of Ace2/Swi5 (C. albicans Ace2), loss of Ace2 leads to attenuation of virulence (Dujon et al. 2004, Kelly et al. 2004). However, in a related species with both orthologues (C. glabrata), deletion of ACE2 leads to hypervirulence (Kamran et al. 2004). This example demonstrates
the importance of these paralogues and the fine-tuning that gene duplication allows (Kellis et al. 2004).

**Localization of Swi5 supports a model for kinase/phosphatase balance in cell cycle control**

CDK phosphorylation of Swi5 excludes it from the nucleus until Cdc14 is released during anaphase (Moll et al. 1991, Visintin R. et al. 1998). Therefore, I investigated Swi5 localization when mitotic cyclins are sustained. Both the timing and amount of Swi5’s initial entry were unaffected by the maintenance of mitotic cyclins, and subsequent nuclear entries revealed oscillations of Swi5 entry during the delay in cell cycle progression. Release of Cdc14 phosphatase from the nucleolus similarly oscillates when mitotic cyclin levels are sustained (Drapkin et al. 2009, Lu and Cross 2010). Swi5 is a mutual target of CDK and Cdc14 phosphatase; therefore, we propose that the repeated Swi5 nuclear entries are a result of the oscillations in the kinase/phosphatase balance between CDK and Cdc14 activities [Figure 2.5].

Overall, these findings support the model we proposed for how cells progress through mitotic exit despite locked mitotic cyclins (Drapkin et al. 2009). In a normal cell cycle, the relevant variable for ordering mitotic exit events may be the balance of competing kinase and phosphatase activities. In support of this model, a mutant in the MEN pathway that cannot release Cdc14 from the nucleolus

- 102 -
(cdc15-2) exits mitosis if the stoichiometric CDK inhibitor SIC1 is overexpressed (Yeong et al., 2000). In a cdc15-2 arrest, the APC\textsuperscript{Cdc20} degrades mitotic cyclin Clb2 to approximately 1/3 peak. The telophase arrest is stable but reverses promptly when Cdc14 is released, even if Sic1 inhibition or APC\textsuperscript{Cdh1} degradation are prevented (Wäsch and Cross, 2002).

In addition to the global activity of the kinase and phosphatase activities, thresholds for particular events in mitotic events may be set by the susceptibility of relevant targets to both the CDK and Cdc14 phosphatase activities. According to preliminary data, the localization of the Swi5 homolog Ace2 may be quite different than that of Swi5 in the presence of sustained mitotic cyclins, demonstrating that very similar proteins may have different responses to the kinase/phosphatase balance. Additionally, neither mitotic cyclin/CDK or Cdc14 phosphatase is distributed uniformly in the cell (Bailly et al. 2003, Buttery et al. 2007, Hood et al. 2001, Pereira and Schiebel 2003, Pereira et al. 2002, Shou et al. 1999, Visintin R. et al. 1999, Yoshida et al. 2002), and therefore, local activity concentrations around substrates may play a role in setting the balance of kinase/phosphatase activity. Accounting for the variables of phosphatase activity, substrate susceptibility, and local concentrations of kinase and phosphatase activities near substrates may explain how the rise and fall of CDK activity once per cell cycle maintains order and timing of events.
Swi5 may promote cytokinesis and bud formation through coordinated promotion of several transcriptional targets

In order to understand the ability of Swi5 to promote cytokinesis and rebudding, I compared the effects of deletions of individual transcriptional targets using our quantitative measurement of mitotic cyclin inhibitory thresholds. Swi5-dependent transcription targets are normally expressed during a time of mitotic cyclin inhibition. However, our quantitative assay may be sensitive to rare regulatory interactions that occur during the transition from high to low CDK activity or mechanisms that are important after checkpoints when mitotic cyclin activity accumulates (Drapkin et al. 2009, Hwang et al. 1998).

So far, individual targets have only had intermediate effects. Aim44 was capable of promoting budding and cytokinesis while Nis1 may only promote cytokinesis. This may indicate that numerous targets of Swi5 coordinate to promote these mitotic events. Some may be involved in both processes while others may be exclusive to an individual process. To fully understand the contribution of this network, combinations of mutants need to be analyzed.

A coordinated effect of Swi5 targets on the subsequent cell cycle events would not be surprising considering the overlapping machinery involved in establishing a new bud and constricting the ring to separate the bud from its mother. Still, some evidence demonstrates that these events may be regulated independently
by CDK activity, including (1) the ability of cells with sustained mitotic cyclin to 
rebud before completing cytokinesis (Drapkin \textit{et al.} 2009) and (2) the ability of at 
least one Swi5 target analyzed, Nis1, to promote cytokinesis, but not bud 
formation [Figure 3.4].

Up until now, the marker used for cytokinesis has been the contraction of the 
actomyosin ring (Myo1-mCherry); however, in yeast, cytokinesis occurs through 
two interdependent pathways (Schmidt \textit{et al.} 2002). Actomyosin ring contraction 
invaginates the plasma membrane; and while constricting, guides the chitin 
synthase Chs2 as it deposits the primary septum (Bi 2001, Bi \textit{et al.} 1998, 
2003). However, if either of these pathways is removed, the cell can still 
complete an aberrant cytokinesis (Schmidt \textit{et al.} 2002, Tolliday \textit{et al.} 2003). At 
least one Swi5 target, Cyk3, is involved in the actomyosin-independent pathway 
and activates Chs2-dependent septum formation (Jendretzki \textit{et al.} 2009, Korinek 
\textit{et al.} 2000, Nishihama \textit{et al.} 2009). Chs2 is restricted to the endoplasmic 
reticulum by CDK phosphorylation and transported to the bud neck during 
be interesting to further explore the role of CDK regulation in these parallel 
pathways by using a marker for the plasma membrane (\textit{e.g.} pleckstrin homology 
domain of phospholipase C fused to GFP (PH–GFP) (Mendoza \textit{et al.} 2009)) to 
score abscission as an additional event in mitotic exit. Furthermore, by analyzing
mutants that eliminate these pathways individually, we can understand the CDK regulation of cytokinesis as a whole.

Aim44 and Nis1 are both implicated in the events of budding and cytokinesis by their localizations and physical interactions, although neither has a defined biochemical activity or well understood function in these processes. Furthermore, searches for sequence or structural homology do not reveal any significantly conserved domains (data not shown). Overall, determining the exact roles of these proteins in the processes of cytokinesis and budding will require significant further analysis.

**Identification of novel Cdc14 phosphatase substrates**

The combination of immunopurification coupled with MS analysis is a powerful tool for identifying physical interactions between proteins. However, interactions between enzymes and their substrates can be brief and hard to obtain with these methods. When trying to identify substrates of the Cdc14 phosphatase, an additional complication of sequestration of the enzyme during most of the cell cycle is introduced. Therefore, Joanna Bloom in our laboratory utilized mutants that constitutively release Cdc14 form the nucleolus or strengthen the interaction between the phosphatase and its substrates to identify novel targets of this key mitotic regulator.
To examine the physiological consequence of Cdc14 dephosphorylation of these targets, I first selected the partially redundant formins Bni1 and Bnr1. These proteins change their localization in the cell at a time coincident with Cdc14 release [Figure 4.5] (Buttery et al. 2007, Gladfelter et al. 2001, Kamei et al. 1998, Ozaki-Kuroda et al. 2001). Showing a direct relationship between these events has been difficult. However, Bnr1 does change localization coincident with an endogenous release of Cdc14 in cells when cell cycle progression is otherwise considerably protracted, suggesting that Cdc14 does directly affect Bnr1 localization [Figure 4.8].

Since septins are known to recruit the formins to the bud neck (Kamei et al. 1998, Pruynne et al. 2004), determining the effect of Cdc14 on this interaction in an in vitro system could further support the hypothesis that this dephosphorylation event promotes a reorganization of formins during anaphase. In the future, identifying the sites of phosphorylation that regulate this exchange would allow us to further elucidate the importance of switching between formins at the bud neck for efficient cytokinesis. However, mutating consensus sites within regions important for bud neck localization did not affect essential protein function (Joanna Bloom).

The combination of determining quantitative relationships and identifying direct substrates of enzymatic activities is powerful in a system as well studied as the
cell cycle. One of the proteins identified as a novel Cdc14 associated protein [Figure 4.1], Nis1, is also a Swi5 target partially responsible for the transcription factor’s ability to promote cytokinesis [Figure 3.4]. Additionally, Sfi1, a component of the SPB bridge, was identified as a likely Cdc14 target (Joanna Bloom; data not shown). Using a similar quantitative assay to the one presented here, our laboratory has shown that CDK phosphorylation of Sfi1 is an important but not rate limiting determinant of spindle formation (Catherine Oikonomou, personal communication). Future work will determine the contribution of additional newly identified substrates to the efficient progression of the cell cycle.
MATERIALS AND METHODS

Strain and Plasmid Construction
Standard yeast methods for strain and plasmid construction were used throughout.

Measuring Mitotic Cyclin Inhibitory Thresholds
(developed by Ben Drapkin; Drapkin et al. 2009)

Loading undegradable mitotic cyclin into metaphase arrested cells.
Strain: ADH1pr-GAL4-rMR cdc20::MET3-HA3-CDC20
with either CLB2::GAL1-CLB2db,ken1,2-YFP or CLB2::GAL1-CLB2db,ken1,2
Protocol:
• Grow in raffinose medium lacking methionine to log phase.
• Incubate for 2.5 hours in 2 mM methionine to turn off MET3-CDC20 and
arrest in metaphase.
• Split culture: pulsed and unpulsed. Induce (pulse) CLB2kd–YFP or
CLB2kd expression for ~30 or ~40 minutes respectively in 10 M
deoxycorticosterone.
• Wash out hormone from media (stop pulse).
• Allow Clb2-YFP fluorescence to mature by additional 45 minutes in
glucose + methionine.
• Wash out methionine from media.
• Release cells in glucose medium lacking methionine.
• If indicated, add 2mM methionine after 45 minutes to block cells at a
second metaphase block.

Population Clb2kd–YFP measurement by immunoblot
Strain: cdc20::MET3-HA3-CDC20 CLB2-YFP
Protocol:
• Grow in glucose medium to log phase.
• Arrest with 100 nM alpha factor (α) for 2.5 hours.
• Release to glucose medium ± methionine.
• Samples collected every 10 minutes. 60 minute timepoints for both
contained equal protein concentrations of Clb2-YFP and the maximum for
the culture without methionine (see below).

Clb2–YFP 60-minute peak and Clb2kd–YFP pulse samples were serially
diluted two-fold into clb2 extract for calibration. Blots were probed with anti-
GFP (Roche), anti-Clb2, and anti-Pgk1 (Santa Cruz Biotechnology) (loading
control) antibodies. ECL signal was imaged using a Fujifilm DarkBox and
CCD camera and quantified using MultiGauge (Fujifilm) software (linear
detection range). Multiple comparisons were performed for each time point.
Quantitative Fluorescence Microscopy

Cells were lightly fixed in 4% paraformaldehyde as described (Drapkin et al. 2009). YFP fluorescence was quantified from single unenhanced exposures, after single-cell masking and background subtraction. This required a mask-making program to define cell regions, developed in MatLab by Ben Timney (Drapkin et al. 2009).

For analysis of spindles, cytokinesis rings and buds, 3–5 0.3 μm, contrast-enhanced optical sections were combined. These procedures yielded single-cell correlations between Clb2kd–YFP levels and mitotic exit phenotypes.

Clb2kd inhibitory threshold calculation

Inhibitory thresholds were measured at different time points after event completion in the unpulsed control.

Clb2kd peak-equivalence (% peak or fraction peak) was determined by:

\[
\text{% Peak} = \frac{F_{\text{Cell}}}{\text{mean } F_{\text{Cell}}} \times \frac{W_{\text{Pulse}}}{W_{\text{Peak}}}
\]

\(F_{\text{cell}}\) = YFP fluorescence of an individual pulsed cell;

\(W_{\text{pulse}}\) the quantified anti-YFP immunoblot signal from the pulsed population;

\(W_{\text{peak}}\) the quantified anti-YFP immunoblot signal from peak synchronized wild-type samples.

Cells were sorted into Clb2kd-YFP bins containing >60 cells, and phenotypes of cells in these bins yielded inhibitory Clb2kd concentrations.

Time-Lapse Microscopy

Preparation of cells for time-lapse microscopy was performed as previously described (Bean et al. 2006).

Adaptations to above threshold measurement protocol (above) for following cells during release by time-lapse microscopy:

- After washing out methionine, cells were transferred to glucose medium without methionine at 30°C and imaged every 3 minutes for 357 minutes.
- For each movie, the fluorescence of a calibrated GFP-conjugated bead is measured. All measurements from that movie are standardized to the bead, to allow comparisons across movies.
- The peak measurement is based on a population average of the maximum YFP fluorescence value of \(MET-CDC20 CLB2-YFP\) cells when arrested in
metaphase by methionine repression for 2.5 hours before imaging. The value was background subtracted and divided by two (two fold relationship between peak Clb2 in a synchronous cell cycle and during a MET-CDC20 block was established by Ben Drapkin, (Drapkin et al. 2009)).

• In movies without the YFP tag on Clb2,kd (e.g. those looking at Swi5 and Ace2 localization), the amount of mitotic cyclin is estimated based on a linear relationship established between the amount of Clb2kd-YFP and the delay in budding (Ying Lu, personal communication).

Image Analysis
Image segmentation and fluorescence quantification were as described (Bean et al. 2006, Charvin et al. 2008).

Immunoblots
Western Blots were performed using standard methods. Antibody concentrations used were mouse monoclonal anti-Pgk1 1:10,000 (Invitrogen), rabbit polyclonal anti-Clb2 1:10,000, and mouse monoclonal anti-GFP 1:1000 (Roche). ECL signal was measured in a Fujifilm DarkBox with CCD camera, and quantified using MultiGauge software (Fujifilm).

Fixed cell fluorescence microscopy
Cells were lightly fixed in 4% paraformaldehyde as described (Drapkin et al. 2009).

Fluorescence and DIC images were acquired using an Axioplan 2 microscope (Carl Zeiss MicroImaging Corp.) with a 63X 1.4 numerical aperture Plan-Apochromat objective, coupled to a Hamamatsu C4742-95 CCD camera (Sciscope Instrument). Camera and microscope were interfaced with the OpenLab software (Improvision). Filters and dichroics used were made by Chroma. YFP was detected with a YFP filter, mCherry with a Cy3 filter, CFP with a CFP filter, and GFP with a narrow band pass FITC filter. Optical sections were taken at 0.3 micron spacing; for illustrative purposes these were merged into two-dimensional maximum projections. Acquisition was automated using an OpenLab script written by Ben Drapkin.
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- 126 -


