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Whi5 Regulation by Site Specific CDK Phosphorylation in
Saccharomyces cerevisiae

A Dissertation submitted in partial satisfaction of the Requirements for the
degree Doctor of Philosophy

in

Biomedical Sciences

by

Michelle Valentina Wagner

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2008
The Dissertation of Michelle Valentina Wagner is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008
DEDICATION

I would like to dedicate this dissertation to my family who have been an inspiration and motivation throughout my graduate experience.

To my husband, Sean Gardinier, for his patience and unwavering support,

To my parents, Bill and Mary Wagner, for always believing in me and teaching me the value of hard work and a job well done,

To my brothers, Joe, Ed, Tom, and Jim Wagner, for instilling in me a healthy sense of competition and teaching me to be tough,

To my sister, Caroline Wagner, the best sister in the whole wide world, for inspiring me and always making me laugh,

I love you, thank you.
EPIGRAPH

The knowledge that you have emerged wiser and stronger from setbacks means that you are, ever after, secure in your ability to survive.

-JK Rowling
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Chapter 7, in part will be submitted for publication in the journal Cell Cycle. Michelle Wagner and Steven Dowdy. The dissertation author was the primary author of this paper.

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ABSTRACT OF THE DISSERTATION

Whi5 Regulation by Site Specific CDK-Phosphorylation in
*Saccharomyces cerevisiae*

by

Michelle Valentina Wagner

Doctor of Philosophy in Biomedical Sciences

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Professor Steven F. Dowdy, Chair

The Whi5 transcriptional repressor is a negative regulator of G1 cell cycle progression in *Saccharomyces cerevisiae* and is functionally equivalent to the retinoblastoma (Rb) tumor suppressor protein in mammals. In early G1, Whi5 binds to and inhibits SBF (Swi4/Swi6) transcriptional complexes. At Start, Cln:Cdc28 kinases phosphorylate and inactivate Whi5, causing its
dissociation from SBF promoters and nuclear export, allowing activation of SBF transcription and entry into the cell cycle. Due to the conserved regulatory pathways controlling G1 transcription between yeast and mammalian cells, investigation into the regulation by phosphorylation of Whi5 in yeast could provide insight into mammalian G1 cell cycle and Rb regulation.

In an analysis of Whi5 phosphorylation, we found that 10 of the 12 putative CDK phosphorylation sites on Whi5 were occupied in vivo in asynchronously growing cells. In addition, we identified 6 non-CDK sites that were phosphorylated. Whi5 CDK and non-CDK phosphorylation mutants were functional and able to rescue the small cell size of whi5Δ cells. However, the Whi5 CDK mutant with all 12 putative CDK sites changed to alanine caused a dramatic cell cycle phenotype when overexpressed with a Swi6 CDK phosphorylation mutant. Mutational analysis of Whi5 determined that only four C-terminal CDK sites were necessary and sufficient for Whi5 inactivation when Swi6 CDK sites were also mutated. Although these four CDK sites did not wholly determine Whi5 nuclear export, they did impact regulation of cell size. Taken together, these observations begin to dissect the regulatory role of specific phosphorylation sites on Whi5.
INTRODUCTION
INTRODUCTION

The Cell Division Cycle

Cell proliferation is a tightly regulated process where extracellular signals and intracellular checkpoints are integrated to control cell growth and division. Cellular checkpoints assessing nutrient availability, cell size, DNA replication and integrity, and chromosomal separation occur during different phases of the mitotic cell division cycle. The cell cycle can be divided into four distinct phases. During G1, gap or growth phase 1, cells sense quality and abundance of extracellular nutrients and begin to increase in size and accumulate mass. When conditions for cell division are favorable, cells commit to enter the cell cycle during G1 phase at an irreversible transition termed Start in yeast and the Restriction Point in mammalian cells (Hartwell et al., 1974; Pardee, 1974). G1 phase is followed by S phase, or synthesis phase, wherein genomic DNA is replicated. A second gap or growth phase, G2, precedes M phase, or mitosis, where chromosomes are divided into two daughter cells. Failure to properly regulate cell cycle entry in G1 or other critical checkpoints throughout the cell cycle can result in abnormal or incomplete division in yeast and can lead to development of neoplastic disease in mammalian cells (Mendenhall and Hodge, 1998). Thus, proper regulation of the cell division cycle is important for overall fitness of both unicellular and multicellular organisms.
Cell size regulation

Within each cell division cycle, a cell must double its mass before dividing into two daughter cells. Coordination of cellular growth with cell division is therefore required to maintain cell size homeostasis over multiple generations. The relationship between cell growth and cell division is important in cell cycle regulation. Inhibition or interference with cell growth results in inhibition of the cell division cycle. Nutrient deprivation or inhibition of TOR, a complex that controls growth in response to nutrients, results in inhibition of cell growth and a subsequent cell cycle arrest (Cardenas et al., 1999; Hartwell et al., 1974). In contrast, inhibition of cell division does not inhibit cell growth, as cells continue to grow and increase in size during most cell cycle arrests (Jorgensen and Tyers, 2004; Saucedo and Edgar, 2002). Therefore, cell growth is required for cell division and regulates cell cycle progression.

In budding yeast, environmental nutrient availability regulates accumulation of cellular mass to a certain cell size during G1 phase prior to Start (Johnston et al., 1977). Yeast cells must attain a critical cell size before they commit to cell division, ensuring there is enough cell mass to form two daughter cells. The critical cell size needed for cell division varies with nutrient quality and abundance. In poor media, cells have a smaller critical size to attain before division but divide at a slow rate. In nutrient poor conditions cells spend more time in early G1 to accumulate mass before cell cycle commitment (Rupes, 2002). When grown in rich media, cells maintain a larger
cell size yet have a faster growth rate. Abundant nutrients allow cells to spend less time in G1 because they quickly accumulate mass and reach the critical cell size. However, the critical cell size required for cell division is dynamic; cells switched from poor media to rich media will increase their cell size before committing to a further round of cell division. This requires an initial delay in G1 to allow growth to the larger size (Flick et al., 2003; Johnston et al., 1979). The ability to alter cell size in varied conditions demonstrates that cells actively sense nutrients and adjust their cell size and rate of cell division accordingly. In mammalian cells, growth factor signaling determines passage through the Restriction Point that is also presumed to be partially responsive to cell size and mass (Pardee, 1974). Thus, regulation of cell size and cell growth before commitment to cell division is conserved between yeast and mammalian cells.

While it is clear that cell growth regulates cell division, the exact mechanism of how this occurs is still under investigation. It is unknown how cells sense their size and control cell growth. Additionally, it is unclear which molecular pathways are utilized to relay the signal of sufficient size to the cell cycle machinery to trigger Start. Recent studies in yeast implicate ribosome biogenesis and rate of synthesis of ribosomal subunits as important factors communicating growth potential and resulting cell size (Jorgensen et al., 2004). When ribosome biogenesis is slowed, cells decrease their size. Thus, rate of ribosome synthesis could be an indicator of nutrient quality and abundance that would impact the set point of critical cell size. It is possible that cells then gauge their size through translation rate, as sublethal dose of
cycloheximide increase the critical cell size (Jorgensen et al., 2004). This implies that cells sense a certain level or rate of translation as a signal of sufficient cell size. The links between ribosome biogenesis, translation rate, and cell cycle regulation may extend to mammalian systems as well. It has been shown that in mice, conditional deletion of a 40S ribosomal protein inhibits cell proliferation but not cell growth (Volarevic et al., 2000), indicating interference in ribosomal assembly affects downstream events promoting cell cycle progression.

**Cell cycle dependent transcription in G1**

Although the mechanisms coordinating cell size with cell cycle commitment remain to be solidified, the pathways acting to transition cells across Start have been well studied. Once cells have accumulated mass and attained the critical cell size needed for cell division, they pass through Start and are committed to progress through the cell cycle. The molecular events determining commitment to cell division in both yeast and mammalian cells involve regulated transcription of groups of genes required for cell cycle progression (Cho et al., 1998; Spellman et al., 1998). Genome wide microarray studies in yeast report cell cycle dependent periodicity for expression of ~800 genes, and greater than 200 genes are specifically activated during G1 phase (Cho et al., 1998; Spellman et al., 1998). Controlled expression of genes involved in cell cycle processes during specific
phases of the cell cycle ensures the gene products are present during the correct interval (Wittenberg and Reed, 1991).

In yeast, induction of gene expression at Start depends on the SBF and MBF transcription factor complexes; each heterodimeric complex consists of the transcriptional coactivator Swi6 and a DNA binding protein, Swi4 or Mbp1, respectively (Breeden, 1996). Both Swi4 and Swi6 have a DNA binding domain near the amino-terminus, a central region of ankyrin repeats, and a region required for binding Swi6 in the carboxy-terminus (Breeden, 1996). SBF binds specific sequences in target promoters termed the Swi4 Cell cycle Box, or SCB elements. Thus SBF is the SCB Binding Factor. Likewise, MBF binds the Mlu1 Cell cycle Box, or MCB, named for the Mlu1 restriction site found in the specific sequence element, making MBF the MCB Binding Factor (see Fig. 0.1 for model).

In general, SBF complexes control transcription of cell cycle regulatory genes (CLN1/2, PCL1/2) and genes involved in cell wall morphogenesis and budding (SVS1, GIN4, FKS1/2) (Iyer et al., 2001; Wittenberg and Reed, 2005). SBF complexes also bind and regulate the promoters of several other transcription factors (HCM1, PLM2, TOS4, YOX1), indicating SBF activation sets off a network of additional transcription (Horak et al., 2002). Functional classes of MBF targets include genes involved in DNA synthesis, recombination, and repair (POL2, RNR1, CLB5/6) (Wittenberg and Reed, 2005). However, both SBF and MBF sites are present in promoters of many
FIG. 0.1. Model of yeast G1 cell cycle progression.
genes, suggesting there is overlap in the specificity of SBF and MBF regulated transcription.

Genetic analysis of SBF and MBF components indicates that they are important for cell cycle progression. Loss of both \textit{SWI4} and \textit{MBP1} results in a permanent G1 arrest and cell lethality, demonstrating the essential function of MBF and SBF transcription factor complexes at Start (Koch et al., 1993). Deletion of \textit{SWI4} and \textit{SWI6} together also results in G1 arrest and cell lethality. Loss of \textit{MBP1} alone results in deregulation of DNA synthesis genes without a severe phenotype. Cells without \textit{SWI4} are also viable, but are slow growing, temperature sensitive, and have low levels of target gene transcripts (Ogas et al., 1991). Both \textit{swi6}\textsuperscript{Δ} single mutant and \textit{mbp1\textsuperscript{Δ}swi6}\textsuperscript{Δ} double mutant cells remain viable, because Swi4 retains some activity as a transcription factor in the absence of Swi6 (Koch et al., 1993). The genetic studies show that SBF and MBF activity is essential at Start, and interference with either complex affects cell cycle progression.

**Regulation of SBF transcription at Start by Cln3**

Given that SBF and MBF complexes control expression of many genes required for cell cycle progression, research has focused on how SBF and MBF activity is regulated. In early G1, SBF and MBF complexes are bound to target promoters, but are repressed. Cells do not activate SBF and MBF transcription until Start (Costanzo et al., 2004; de Bruin et al., 2004). Proper
timing of transcriptional activation at Start once cells reach a critical cell size requires the G1 cyclin Cln3 and the cyclin dependent kinase (CDK) Cdc28 (Dirick et al., 1995; Tyers et al., 1993). Cln3 is a dose dependent activator of cell cycle progression. Cells lacking \textit{CLN3} have a large cell size phenotype and are delayed in transcriptional activation and passage through Start. Cells with extra copies of \textit{CLN3} are smaller, pass quickly through Start, and are resistant to mating pheromone arrest in G1 (Cross, 1988; Dirick et al., 1995; Tyers et al., 1993). These observations support the idea that Cln3 is a positive regulator of G1 important for timing of transcriptional activation.

Although timing of Start is sensitive to Cln3 dosage, it is unknown how Cln3-Cdc28 activity is regulated at Start to induce a rapid and robust activation of SBF and MBF. Cln3 protein levels and Cln3-Cdc28 associated kinase activity do not increase during G1 phase, and are fairly constant throughout the cell cycle (Tyers et al., 1993). Therefore, several models of Cln3 regulation at Start have been proposed, including regulation by phosphorylation and proteolysis (Tyers et al., 1992). Two additional proposed mechanisms regulating Cln3 cellular localization might explain Cln3 regulation at Start. Wang \textit{et al.} demonstrate that Cln3-Cdc28 complexes are localized in the cytoplasm of cells in early G1, retained in complexes with a negative regulator Whi3, and become nuclear at Start and in late G1 (Wang et al., 2004). Cln3 has also been shown to be sequestered at the endoplasmic reticulum in early G1, and released by the chaperone Ydj1 to allow timely entry into the cell cycle at Start (Verges et al., 2007). Thus, an increase in
nuclear abundance of Cln3 once cells reach a critical cell size may contribute to transcriptional activation at Start.

Although Cln3 is required for timely activation of Start when cells reach a critical cell size, CLN3 is not essential for cell viability. In the absence of CLN3, the other G1 cyclins Cln1 and Cln2 are necessary and sufficient for activation of SBF and MBF transcription (Dirick and Nasmyth, 1991). CLN3 is essential for viability, however, when CLN1 and CLN2 are inactivated, meaning the Clns are an essential cyclin family (Richardson et al., 1989). CLN3 also becomes essential in cells lacking BCK2, a poorly understood activator of Start that functions in parallel with Cln3. Bck2 functions partially independent of SBF and MBF to promote Start, and is essential in cells lacking SWI4 or SWI6 (Wijnen and Futcher, 1999). The activities of Bck2 and Cln3 together are essential for the cell cycle to pass through Start.

As a positive regulator of cell cycle progression, Cln3-Cdc28 kinase activity has several functions at Start. Cdc28 activity is required to recruit RNA polymerase PolII, TFIIB, and TFIIH to SBF and MBF complexes to complete formation of the RNA polymerase holoenzyme and promote transcription (Cosma et al., 2001). Additionally, Swi4 and Swi6 are also known targets of CDK phosphorylation and genetic studies implicate Swi6 as a critical target of Cln3 (Wijnen et al., 2002). The functions of Cln3 including control of cell size, mating pheromone arrest, and cell cycle regulated transcription, all require a functional SWI6 (Wijnen et al., 2002). However, mutation of multiple CDK phosphorylation sites of Swi4 or Swi6 does not interfere with timing of SBF
transcription, indicating their phosphorylation is not essential for transcriptional activation (Koch et al., 1996; Sidorova et al., 1995; Wijnen et al., 2002). Two studies by Costanzo et al. and de Bruin et al. describe an additional substrate of Cln3-Cdc28 phosphorylation at Start, the transcriptional inhibitor Whi5 (Costanzo et al., 2004; de Bruin et al., 2004).

**Inhibition of SBF transcription by Whi5**

Whi5 is a key target of Cln3-Cdc28 cyclin dependent kinase activity that promotes transcriptional activation of SBF. Whi5 is bound to SBF at promoters and functions to repress transcription in early G1 (Costanzo et al., 2004; de Bruin et al., 2004). Whi5 is then released from promoters and exported to the cytoplasm at Start, allowing SBF activation (Costanzo et al., 2004). Inactivation of Whi5 occurs by phosphorylation of Whi5 and SBF complexes by Cln3-Cdc28, resulting in induction of G1 transcription at Start (Costanzo et al., 2004; de Bruin et al., 2004). Whi5 dissociation from SBF promoters and subsequent nuclear export correlates with timing of induction of SBF transcription and cell cycle entry (Costanzo et al., 2004; de Bruin et al., 2004). Cells lacking CLN3 lose tight control of Whi5 inactivation, releasing Whi5 from SBF promoters in a gradual and delayed fashion (de Bruin et al., 2004). While cells lacking both CLN1 and CLN2 are still able to promote Whi5 release from SBF promoters in a timely fashion, cln1Δcln2Δcln3Δ cells permanently arrest in G1 with Whi5 bound to SBF (de Bruin et al., 2004).
Thus, Cln-Cdc28 activity is required for Whi5 inactivation and SBF activation, and specifically Cln3-Cdc28 is required for proper timing of these events.

Genetic analysis of Whi5 reveals it is a negative regulator of cell cycle progression. In a global screen of yeast deletion mutants that effect cell size regulation, \textit{whi5}Δ cells were found to have a small cell size phenotype (Jorgensen et al., 2002). Accordingly, cells lacking \textit{WHI5} initiate SBF transcription prematurely, driving cells past Start and resulting in a small cell size (Jorgensen et al., 2002). Overexpression of Whi5 from the \textit{GAL1} promoter results in a large cell size and a cell cycle delay in G1 phase (Costanzo et al., 2004). Thus, Whi5 appears to act as a dose dependent inhibitor of Start, much like Cln3 is a dose dependent activator.

Interestingly, in epistasis tests \textit{cln3}Δ\textit{whi5}Δ cells are small in size like \textit{whi5}Δ cells, not large like \textit{cln3}Δ cells, indicating Whi5 is downstream of Cln3 activity (Costanzo et al., 2004; de Bruin et al., 2004). In combination with the other G1 activator, Bck2, epistasis of Whi5 and Bck2 was intermediate. \textit{whi5}Δ\textit{bck2}Δ cells are almost normal cell size, not small like \textit{whi5}Δ cells or larger like \textit{bck2}Δ cells (Costanzo et al., 2004), indicating Bck2 does not necessarily function through inhibition of Whi5 function. However, de Bruin et al. show that Whi5 is the only essential target of Cln3 and Bck2. Cells lacking both \textit{CLN3} and \textit{BCK2} are lethal, permanently arresting in G1. Subsequent inactivation of \textit{WHI5} in those cells rescues the lethality, making triple knockout \textit{cln3}Δ\textit{bck2}Δ\textit{whi5}Δ cells viable and able to pass Start and complete cell division (de Bruin et al., 2004). Deletion of the Whi5 inhibitor in cells lacking the two
activators of G1 is able to rescue the cell cycle defect and support viability. These observations place Whi5 as a chief inhibitor of G1 cell cycle progression at Start, responsive to inactivation by Cln3 and downstream of pathways that promote cell size accumulation.

**Redundant phosphorylation of Whi5 and Swi6**

While CDK activity is essential for SBF transcriptional activity, and Whi5 is an essential target of G1 activators Cln3 and Bck2, phosphorylation of Whi5 is not essential for cell cycle progression. Expression of a mutant allele of Whi5 with all 12 of its putative CDK phosphorylation sites mutated to alanine (Whi5-12Ala) does not affect cell division (Costanzo et al., 2004). The CDK sites of Whi5 are also not required for its release from SBF complexes. Costanzo, *et al.* demonstrated that *in vitro*, Whi5 dissociated from SBF complexes of Swi4 and Swi6 upon addition of active Cln2-Cdc28. However, Whi5-12Ala was still able to dissociate from Swi4 and Swi6 after kinase addition; thus, the dissociation of Whi5 from SBF did not depend on Whi5 CDK sites (Costanzo et al., 2004). This result implies that phosphorylation of Swi6 or Swi4 is inducing Whi5 dissociation from SBF. Interestingly, the Whi5-12Ala CDK phosphorylation mutant causes cell lethality when overexpressed in a Swi6 CDK phosphorylation mutant background, suggesting that CDK phosphorylation of Whi5 and Swi6 is redundant for transcriptional activation (Costanzo et al., 2004).
While the Swi6 CDK mutant does not significantly affect transcriptional activation on its own (Sidorova et al., 1995), it causes a dramatic cell cycle inhibition when Whi5-12Ala is overexpressed. It is not known how Swi6 CDK sites are important for transcriptional activation, but it has been demonstrated that Swi6 is also subject to inhibitory phosphorylation. During the late G1 to early S-phase transition, Swi6 is phosphorylated by cyclin Clb6-Cdc28 on Ser-160, that is located near the Swi6 nuclear localization sequence. This phosphorylation inhibits nuclear import, changing Swi6 cellular localization from nuclear to cytoplasmic (Geymonat et al., 2004; Sidorova et al., 1995). This serves to inactivate SBF transcription outside of G1. It is unknown if the cell cycle inhibitory effect observed with overexpression of Whi5-12Ala along with the Swi6 CDK mutant depends on Swi6 Ser-160, or if additional CDK sites of Swi6 are important.

**Current model of budding yeast G1 cell cycle progression**

In general, cell cycle progression through G1, S, G2, and M phases is controlled by cyclin-CDK complexes that positively regulate the cell cycle by phosphorylating a variety of targets. Cell division is also inhibited by negative regulators functioning to repress cell cycle progression at various checkpoints. In early G1, SBF and MBF complexes are bound to target promoters but do not activate transcription (See Fig. 0.1 for cell cycle model). SBF is inhibited by Whi5 binding; however, it is not currently known what mechanisms repress MBF in early G1. In response to extracellular nutrients, cells increase in mass
and attain a critical cell size (Johnston et al., 1977). Once at a sufficient size, proper timing of SBF and MBF activation requires Cln3-Cdc28 (Tyers et al., 1993). Whi5 and SBF components are phosphorylated, activating SBF transcription at Start. It is currently unknown what serves to activate MBF at Start.

Cell cycle regulated transcription at Start induces expression of cyclins Cln1/2 and Clb5/6. Clb5 and Clb6 are inhibited in late G1 by the cyclin-CDK inhibitor Sic1. As cells progress through late G1, Sic1 is phosphorylated on multiple sites by Cln1/2-Cdc28 and targeted for degradation (Deshaies and Ferrell, 2001; Nash et al., 2001). This allows for activation of Clb5/6-Cdc28 and promotes entry into S phase. After SBF transcription occurs, both Swi6 and Swi4 dissociate from promoters to inhibit continued transcription outside of G1 phase (Koch et al., 1996). Swi6 is exported to the cytoplasm, retained outside of the nucleus by Ser-160 phosphorylation (Sidorova et al., 1995). Conversely, MBF remains on target promotors through the rest of the cell cycle. The MBF target Nrm1 is responsible for repression of MBF upon exit from G1 (de Bruin et al., 2006).

**Current model of mammalian G1 cell cycle progression**

The basic framework of the cell cycle machinery is conserved between yeast and mammalian cells. Cyclin-CDK complexes activating cell cycle progression balanced by inhibitory factors controlling cell cycle checkpoints is a common theme. Additionally, regulated transcription of groups of genes to
activate cell cycle progression is a basic tenet of both systems. Thus, the system of transcriptional regulation in G1 that occurs in yeast also is present in a functionally similar system in mammalian cells (See Fig. 0.2 for cell cycle model).

Like SBF and MBF, the E2F family of transcription factors controls expression of a suite of E2F target genes essential for late G1 cell cycle progression and DNA synthesis (Sun et al., 2007). In G0 or quiescent cells, E2Fs are assembled on promoters but bound and repressed by the pocket protein p130. In response to growth factor stimulation, Cyclin D-Cdk4/6 is activated and partially phosphorylates the retinoblastoma tumor suppressor protein (Rb), a functional analogue of Whi5 (Harbour and Dean, 2000; Kaelin, 1999). The hypo-phosphorylated form of Rb binds and represses E2Fs in early G1 (Ezhevsky et al., 1997). At the restriction point, activation of Cyclin E-Cdk2 kinase complexes results in Rb hyper-phosphorylation and inactivation, allowing for cell cycle-dependent E2F transcription (Ho and Dowdy, 2002). E2F target genes include Cyclin E, genes for cell metabolism such as DHFR, and genes involved in regulating DNA synthesis such as Cyclin A.

Cyclin A mRNA transcript is induced in late G1 when E2F transcription is activated. Cyclin A protein, however, does not accumulate until just before S phase. Cyclin A mRNA is translated in late G1; thus, Cyclin A levels are regulated at the protein level. Cyclin A protein is ubiquitinated and targeted for degradation by APC$^{Cdh1}$ in late G1 (Peters, 2002). A recent study
FIG. 0.2. Model of mammalian G1 cell cycle progression.
demonstrated that physiologic levels of endogenously generated reactive oxygen species, or ROS, serve to inactivate APC^{Cdh1} in late G1 just before S phase. The inhibition of APC^{Cdh1} allows accumulation of Cyclin A and other proteins required for DNA replication (Havens et al., 2006).

**Regulation of Rb and Whi5 by phosphorylation**

Studies of Rb regulation by phosphorylation show that Rb is phosphorylated at low levels, or hypo-phosphorylated, when bound to E2Fs in early G1 (Ezhevsky et al., 1997); however, it is unknown if the hypo-phosphorylation is required for Rb binding of E2F. Additionally, it has been proposed by some groups that specific CDK sites on Rb are utilized for inactivation (Kitagawa et al., 1996; Knudsen and Wang, 1996), while others suggest a model where a critical threshold of phosphorylation on any of Rb’s 16 CDK sites induces inactivation (Brown et al., 1999; Ezhevsky et al., 2001). Despite the many investigations analyzing Rb function, the mechanistic understanding of Rb regulation by phosphorylation remains unclear. Due to the conserved regulatory pathways controlling G1 transcription between yeast and mammalian cells, investigation of regulation by phosphorylation of Whi5 in yeast could provide insight into mammalian G1 cell cycle and Rb regulation.

In this study we perform an analysis of Whi5 phosphorylation to understand how phosphorylation of the transcriptional inhibitor influences cell cycle progression. We determined that, like Rb, Whi5 is also found hypo-phosphorylated in early G1, but that hypo-phosphorylation is not required for
Whi5 function. Whi5 phosphorylation mutants were able to control cell size and interact genetically with other regulators of Start in a similar manner to wild type Whi5. Additionally, we identified four specific CDK sites in Whi5 that are critical for Whi5 inactivation when Swi6 phosphorylation is also prevented, supporting previous data that phosphorylation of Whi5 and Swi6 is redundant. Although the four specific CDK sites of Whi5 we identified as critical for Whi5 inactivation do not fully support Whi5 nuclear export, they are required for proper regulation of cell size in combination with Swi6 CDK sites. These results demonstrate that regulation of Whi5 by phosphorylation relies on specific CDK sites. Because the regulatory pathways of cell cycle entry are largely conserved between yeast and mammalian cells, the mechanism of regulation by specific phosphorylation observed for Whi5 suggests further investigation of Rb phosphorylation in mammalian systems is warranted.
CHAPTER 1

MATERIALS AND METHODS
**Yeast Culture**

Cells were grown in standard media containing yeast extract, peptone, and 2% glucose at 30°C. For galactose induction experiments, cells were grown overnight in media containing 2% raffinose then inoculated into 2% galactose media or galactose plates. For methionine induction, cells were grown in media with a 2x excess of methionine then inoculated or plated on media without methionine. For reactive oxygen species (ROS) experiments, stocks (1 M, in water) of Tempol (Calbiochem) and 4-hydroxy-Tempo (Sigma), identical compounds that are free radical scavenger/spin traps, were used at final concentrations of 50 mM. Tempamine (Sigma) was used at a 15 mM concentration diluted from a 1 M stock in water. Tempo (Sigma) was used at 3-3.5 mM concentration diluted from a 500 mM stock in 50% ethanol.

**Yeast Strains and Genetic Manipulations**

The Mat a haploid wild type parent strain used in this study, BY4741, is a derivative of *Saccharomyces cerevisiae* S288C. Strains containing open reading frame knockouts are from the *Saccharomyces* Genome Deletion Project (Open Biosystems). To make double knockout cells, the method of Brachmann et al was used (Brachmann et al., 1998). A 13x-Myc tag or a 3x-HA tag was appended to *WHI5* using the method of Longtine et al (Longtine et al., 1998). The method of Longtine et al was also used to append a 13xMyc tag to the C-terminus of *CLN1, CLN2, CLB5, SIC1*, and *PCL1*. The *WHI5* promoter construct derived from 545 base pairs 5' of the *WHI5* open reading
frame was PCR amplified from genomic DNA and cloned into the pRS413 vector (Brachmann et al., 1998). For GFP, Myc, or HA tagged plasmid constructs, the *WHI5* stop codon was mutated to a *Spe1* restriction site and the tag plus the *ADH1* terminator cassette from Longtine et al was inserted in frame (Longtine et al., 1998).

See Table 1.1 for a list of yeast strains used in this study.

**Plasmid Mutagenesis**

The method of the Quick Change Site-Directed Mutagenesis Kit (Stratagene) was used to introduce mutations into the *WHI5* open reading frame. Primers (Integrated DNA Technologies) were designed to change the serine or threonine codon for a CDK or non-CDK phosphorylation site to an alanine codon, or from an alanine back to a serine or threonine. In cases where CDK or non-CDK sites were in close proximity, mutation of both sites was accomplished with one set of primers. Primers included 19-20 bases on either side of the codon(s) to be mutated. PCR reactions with PfuUltraII (Stratagene) were performed for 16 cycles with amplification at 68°C. Reactions were digested with *DpnI* restriction enzyme (New England Biolabs) at 37°C for at least one hour to eliminate un-mutated plasmid DNA before transformation into competent *E. coli* (Stratagene). Subsequent mutant plasmids were sequenced (Eton Bioscience) to verify intended mutated DNA sequence.
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**Plasmid Transformation**

Log phase cultures were washed with sterile water and aliquoted into sterile tubes. Cells were resuspended in 50 µL of 2 mg/mL denatured sonicated salmon sperm DNA (Stratagene) and 34 µL of 4 µg of plasmid DNA diluted in H₂O, prepared with QIAPrep Spin Miniprep Kit (Qiagen), or 34 µL of PCR product. To the suspended cells, 240 µL 50% Polyethylene Glycol and 36 µL 1 M Lithium Acetate was added. Cells in the transformation mixture were incubated for 30 minutes at 30°C then for 25 minutes at 42°C. Cells were spun down and resuspended in complete media before plating on selection media. For cassettes requiring selection on G418 (Gibco), transformation reactions were plated on rich media for one day before replica plating to plates containing 200 mg/mL G418.

**Protein Purification**

Cells with plasmids GAL1-WHI5-3xHA or GAL1-WHI5-12Ala-3xHA were grown in raffinose then induced with galactose for ten hours. Cells from twelve liters of culture were harvested by centrifugation, resuspended in 5 mL of water to make a paste, and drop frozen in liquid nitrogen. Frozen cells were broken with six cycles in a Waring blender in liquid nitrogen, refilling the liquid nitrogen after each cycle. Resulting powder was resuspended in RIPA buffer with protease (PMSF, aprotinin, leupeptin, benzamidine) and phosphatase inhibitors (Sigma cocktails 1 and 2) and incubated for 30 minutes on ice. Cell debris was pelleted by high-speed centrifugation. Cell lysates were subject to
immunoprecipitation with anti-HA affinity resin (Roche) that was performed overnight. Immunoprecipitates were washed with RIPA buffer and boiled in SDS-sample buffer. The boiled samples were then resuspended in 20x volume of RIPA buffer, to dilute the SDS to 0.1%, before a second immunoprecipitation was performed. Samples from the double immunoprecipitation were separated by 10% SDS-PAGE and stained with Coomassie blue.

**Mass Spectrometry**

(This experiment was performed by Dr. Marcus Smolka.)

After in-gel digestion, phosphopeptides were purified by IMAC as previously described (Smolka et al., 2005). Purified phosphopeptides were analyzed by µLC-ESI-MS/MS on a Thermo Finnigan LTQ quadrupole ion trap mass spectrometer as described (Smolka et al., 2005). For data analysis, SEQUEST (version 3.4 beta 2) program running on a Sorcerer system (SageN, San Jose, CA) was used for peptide identification. Database search was performed using the budding yeast database. The following variable modifications were considered: +80 Da (phosphorylation) for serine, threonine and tyrosine residues; +16 Da (oxidation) for methionine residues. Up to 4 variable modifications were allowed per peptide and peptide mass tolerance used was 3 Da. A semi-tryptic restriction was applied, and only the top-matched peptides with a probability score above 0.9 were subsequently considered for close inspection. Each MS/MS spectrum that led to
phosphopeptide identification was manually verified to confirm all significant ions were accounted for, and then validated.

**Cell Synchronization**

For arrest in early G1 phase, cells in log phase growth were washed, resuspended in fresh media containing 5 µg/mL alpha factor (Sigma), and incubated for three hours at 30°C (Amon, 2002). Cells were pelleted and frozen in dry ice, and then stored at -80°C until subsequent use. Alternatively, to release, cells were washed twice with cold media then inoculated into warm media. At indicated time points, samples were taken, cooled with ice, sonicated, then analyzed for budding index. For some experiments samples were also washed and frozen for subsequent RNA extraction and RT-PCR. For arrest in Mitosis, 5 µg/mL nocodazole (Sigma) in DMSO was added to cells in log phase growth and cells were incubated for three hours at 30°C. Cells were pelleted and frozen in dry ice, and then stored at -80°C until subsequent use.

**SDS-PAGE and Immunoblotting**

Protein samples were boiled in 1x SDS loading buffer for five minutes before loading on an 8-10% gel, depending on the molecular weight of the protein being analyzed. Electrophoresis was done at 100 V until the dye front of the gel reached the bottom. After separation, the gel was incubated in transfer buffer before semi-dry transfer to nitrocellulose membrane.
Immunoblots were blocked with 6% dry milk in PBS/0.1% Tween-20 before incubation with primary antibody. Antibodies were diluted 1:1000 in 6% dry milk PBS/0.1% Tween-20 and incubated at least one hour. Secondary HRP conjugated antibodies (Santa Cruz Biotechnology) were used at 1:1000 dilutions. Signal from Enhanced Chemiluminescence Super Substrate (Thermo) was detected with Biomax MR Film (Kodak). Primary antibodies used include anti-Myc (Santa Cruz Biotechnology), anti-HA (Covance), anti-CDC28 (Santa Cruz Biotechnology), and anti-β tubulin (Santa Cruz Biotechnology).

**Protein Analysis with Phosphatase Treatment**

Cells were broken with glass beads in 50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% NP-40 lysis buffer containing protease (PMSF, aprotinin, leupeptin, benzamidine) and phosphatase inhibitors (Sigma phosphatase inhibitors cocktails 1 and 2, diluted 1:100). Immunoprecipitation was performed with anti-Myc antibody (Santa Cruz) and protein G beads (Zymed) or anti-HA conjugated beads (Roche). After washing with lysis buffer without phosphatase inhibitors, and 50 mM Tris, pH 8, 50 mg/mL BSA, 25 uM DTT buffer, immunoprecipitates were incubated with 3 µL lambda protein phosphatase (New England Biolabs) or 3 µL protein phosphatase 1, PP1 (New England Biolabs) in supplied buffers for 1 hour at 30°C (Flick et al., 2003). Analysis of protein migration utilized 10% SDS-PAGE run for 10-12cm, followed by Immunoblotting.
Protein Analysis with IEF-2D gel electrophoresis

Cells were lysed and proteins immunoprecipitated with anti-Myc antibody as above. Immunoprecipitates were washed with 1 mM EGTA, 75 mM KCl, 50 mM Tris, pH 8.5, then washed with 50 mM Tris to get rid of salts. Protein was eluted in 8 M Urea, 50 mM Tris, pH 8.5, 4% CHAPS. Immobiline Dry Strip gels (7 cm, pH 3-10) (GE Healthcare) were rehydrated in 75 µL of 8 M Urea, 2% CHAPS, 2% IPG Buffer (GE Healthcare) combined with 50 µL protein sample. Isoelectric focusing was done by electrophoretic separation with increasing voltages stepwise from 200 to 3,500 V for 3 hours. For the second dimension, strips were equilibrated in 6 M Urea, 75 mM Tris, pH 8.8, 30% glycerol, 2% SDS, 0.002% Bromophenol Blue with 10 mg/mL DTT for 1 hour for first equilibration, then with 25 mg/mL iodoacetamide for second equilibration. This was followed by 9% SDS-PAGE and immunoblotting with anti-Myc antibody (Santa Cruz Biotechnology).

Whole Cell Orthophosphate Labeling

(This experiment was performed in part by Dr. Steven Dowdy.)

Cells were grown overnight in low-phosphate synthetic media containing yeast nitrogen base without phosphate, 2% raffinose, and 0.1 mM KH₂PO₄. They were diluted in the same media plus 2% galactose and incubated 4 hours. Cells were washed and resuspended in 1 mL galactose media without phosphates. 1 mCi ³²P-ortho-phosphate (MP Biomedicals) was
added to each sample and incubated for 2 hours at 30°C. Cells were washed, pelleted, and frozen in dry ice before proceeding with lysis with glass beads in RIPA buffer and double immunoprecipitation as described above. Samples of double immunoprecipitates were run on SDS-PAGE and transferred to nitrocellulose. The membrane was exposed on a Phosphor Screen (Molecular Dynamics) for 24 hours then subject to immunoblotting. The Phosphor Screen was developed on a Phosphor Imager (Molecular Dynamics) and analyzed with ImageQuant v1.1 software (Molecular Dynamics).

**Cell Size Measurements and Determination of Cell Number**

Cell size analysis was performed on asynchronous cultures during log-phase growth using a Coulter Z2 Particle Cell Analyzer (Beckman-Coulter). Cultures were sonicated for 30 seconds before analysis. Cell size distribution was analyzed with the Z2 AccuComp software (Beckman-Coulter). For experiments testing cell number, cells were sonicated, diluted 1:100 in saline solution, and counted using the Coulter Z2 Particle Cell Analyzer (Beckman-Coulter).

**Co-Immunoprecipitation**

Cells were lysed in 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 0.2% NP-40, containing protease (PMSF, aprotinin, leupeptin, benzamidine) and phosphatase inhibitors (Sigma cocktails 1 and 2). Lysates were precleared with protein G beads (Zymed) before addition of 2 μg anti-
Myc antibody (Santa Cruz Biotechnology) or anti-HA resin (Roche) and incubation at 4°C on a rotator for 2 hours. Protein G beads were added to anti-Myc samples for an additional hour. Beads were washed three times with lysis buffer before boiling with SDS sample buffer and proceeding with SDS-PAGE.

**Real-Time RT-PCR and Microarray Analysis**

Total RNA was isolated from cells with the RNeasy Kit (Qiagen). The QuantiTech SYBR Green RT-PCR kit was used for RT-PCR experiments (Qiagen). Reactions were done on a Chromo-4 qPCR system (MJ Research) using standard RT-PCR conditions. Data analysis was performed with MJ Opticon Monitor Analysis Software 3.0 (MJ Research).

For microarrays, duplicate samples of RNA from untreated and Tempol treated cells were applied to the Yeast Genome 2.0 Array (Affymetrix). Data was analyzed with Gene Spring 7.2 Software (Silicon Genetics). The average of the duplicate samples was used in analysis.

**Microscopy**

Fluorescence and differential interference contrast microscopy was performed on live cells with a DeltaVision RT Microscope (Applied Precision Life Science) using a 100x oil objective. Image processing was done with ImageJ software (National Institutes of Health) and fluorescent and DIC images were overlaid using Adobe Photoshop. For quantification, both
budded and unbudded cells were counted and assessed for localization of Whi5-GFP. If distinct nuclear accumulation was observed, localization was deemed nuclear. Localization was deemed cytoplasmic only when diffuse Whi5-GFP signal was observed throughout the cell.

**Flow Cytometry**

Cell cycle profiles were analyzed on a Beckton Dickinson FACScan. Before FACS analysis, cells were washed with cold water and fixed in 1 mL 70% ethanol. After washing with 50 mM NaCitrate, cells were treated with 250 µg/mL RNaseA (Sigma) for one hour and 10 µg/mL ProteinaseK (Roche) for an additional hour. Cells were sonicated for 20 seconds then incubated with propidium iodide (16 µg/mL) in 50 mM NaCitrate for at least 30 minutes. For DNA profiles, 10,000 cells were counted and percentage of cells in G1, S, G2/M determined with FACScan software. For cell size determination, the mean forward scatter-height (FSC-H) was measured. For quantification of intracellular ROS levels, live cells were incubated with dihydroethidium (Calbiochem) and measured for fluorescence.

**Immunoblotting and Kinase Assays for ROS experiments**

Cells were lysed by vortexing with glass beads in 50 mM Tris, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, and protease and phosphatase inhibitors (Sigma). Anti c-Myc (9E10), Cdc28 (yC-20, SantaCruz Biotechnology), and alpha-tubulin (B512, Sigma) antibodies were used for
Western Blots. For kinase assays, Myc-tagged cyclin was immunoprecipitated with 10μg of anti-c-Myc antibody. Kinase reactions were performed in 50 mM Hepes, pH 8.0, 10 mM MgCl₂, 50 μM ATP, and 3 μCi γ³²P-ATP using 2 μg of histone H1 as substrate. Reactions were done at 30°C for 30 minutes.
CHAPTER 2

Whi5 Phosphorylation *in vivo*
The transcriptional repressor Whi5 is a known target of CDK phosphorylation. Recombinant Whi5 can be phosphorylated by Cln3-Cdc28, Cln2-Cdc28, and Clb5-Cdc28 in vitro, indicating that it is a promiscuous CDK substrate (Costanzo et al., 2004). Whi5 also becomes increasingly phosphorylated as cells progress through the cell cycle, as a slower migrating form of Whi5 is seen to accumulate in immunoblot analysis (de Bruin et al., 2004). Phosphorylation of Whi5 correlates with its release from SBF promoters, indicating that CDK regulation of Whi5 is important in its function. Indeed, in *in vitro* experiments, CDK activity induces dissociation of Whi5 from Swi4 and Swi6 SBF complexes (Costanzo et al., 2004; de Bruin et al., 2004). Additionally, *in vivo*, Whi5 does not dissociate from SBF promoters when Cln-Cdc28 activity is inhibited. CDK phosphorylation is also important in Whi5 nuclear export, as a Whi5 mutant with 6 C-terminal CDK sites mutated to alanine remains in the nucleus, while wild type Whi5 is shuttled to the cytoplasm after Start (Costanzo et al., 2004).

There are other intriguing elements about Whi5 phosphorylation. First, Whi5 readily co-immunoprecipitates with kinase activity from wild type cells. Whi5 also co-immunoprecipitates with kinase activity from cdc28-4 cells encoding a temperature sensitive mutant of Cdc28 that is inactive at the restrictive temperature (Costanzo et al., 2004). Although the amount of Whi5 associated kinase activity is less in cdc28-4 cells in comparison to CDC28
cells, this suggests that kinases other than Cdc28 associate with Whi5 and are capable of targeting it for phosphorylation.

Additionally, analysis of Whi5 migration in SDS-PAGE has given insight into its phosphorylated state. When purified from asynchronous cells, a species of reduced mobility of Whi5 is observed in addition to a species of greater mobility. Upon phosphatase treatment, the slower migrating and faster migrating species are eliminated and Whi5 migrates as a 3rd, even faster migrating band (de Bruin et al., 2004). Samples treated with phosphatase and phosphatase inhibitors did not change in migration status, proving the change in migration after phosphatase treatment was due to removal of phosphate groups. This result suggests that Whi5 is always phosphorylated to some extent in cells, and the un-phosphorylated form of Whi5 does not occur in vivo.

In this section, we describe results of Whi5 mass spectrometry analysis that identified phosphorylated residues of Whi5. We provide data demonstrating that non-CDK kinases phosphorylate Whi5. We also present data detailing the phosphorylation status of Whi5 phosphorylation site mutants. A preliminary understanding what phosphorylation sites of Whi5 are occupied facilitates the further analysis of the function of Whi5 phosphorylation.
RESULTS

Whi5 is phosphorylated on 16 CDK sites *in vivo*

Whi5 contains 12 putative CDK phosphorylation sites typified by Ser/Thr-Pro(-X-Basic). The primary amino acid sequence of Whi5 is shown in Fig. 2.1, with putative CDK sites highlighted in blue. Four putative CDK sites in Whi5, numbers 1, 2, 11, and 12, are perfect CDK consensus sites having a spacer and a basic residue following the Ser/Thr-Pro. The other 8 putative CDK sites, numbers 3-10, are minimal consensus sites composed of Ser/Thr-Pro and are not followed by a basic residue (Fig. 2.1). Phosphorylation of Whi5 by Cln:CDK activity has been shown to be important in Whi5 inactivation (Costanzo et al., 2004; de Bruin et al., 2004). de Bruin et al. previously determined that CDK sites 2, 4, 5, 10 and 12 were phosphorylated *in vivo* (de Bruin et al., 2004).

To identify Whi5 phosphorylation sites *in vivo* in a comprehensive manner, we performed mass spectrometry analysis of Whi5. Wild type Whi5 containing a 3x HA C-terminal tag under the control of the GAL1 promoter was immunopurified from asynchronously growing cells with a double immunoprecipitation. The purified protein was digested with trypsin and phosphopeptides were purified by IMAC resin liquid chromatography enrichment then analyzed by mass spectrometry. Ten of the 12 putative Whi5 CDK phosphorylation sites, numbered 3-12, were identified as being phosphorylated (Fig. 2.1), while phosphorylation of sites 1 and 2 were not
FIG. 2.1. Whi5 is phosphorylated on 16 sites \textit{in vivo}.
The primary amino acid sequence of Whi5 is shown with putative CDK sites boxed in blue. Arrows indicate amino acids found phosphorylated \textit{in vivo}. Non-CDK sites phosphorylated in vivo are boxed in green.

Mass Spectrometry analysis of Whi5 was performed by Dr. Marcus Smolka in the laboratory of Dr. Huilin Zhou, UCSD.
detected. These results confirm that Whi5 is a substrate of CDK activity in vivo, and expand the number of known occupied CDK sites.

**Whi5 is phosphorylated on 6 non-CDK sites in vivo**

Surprisingly, in addition to CDK sites, we also identified 6 other non-CDK serine and threonine residues phosphorylated on Whi5, labeled A to F and highlighted in green in Fig. 2.1. We note that 5 of the 6 non-CDK phosphorylation sites contain an acidic residue distal to the phosphorylated Ser/Thr, suggesting that a single class of kinases may be responsible for targeting those sites. Mass spectrometry analysis of purified mutant Whi5 with all 12 CDK sites changed to alanine (Whi5-12Ala-3xHA) maintained phosphorylation of these 6 non-CDK sites. This indicates non-CDK phosphorylation is not dependent on previous CDK phosphorylation. Whi5 contains 4 tyrosine residues, but no tyrosine phosphorylation of Whi5 was detected. In total, we identified 16 in vivo phosphorylation sites on Whi5 in asynchronously growing cells.

**Whi5 is phosphorylated at all stages of the cell cycle.**

We next analyzed the cell cycle dependency of Whi5 phosphorylation. In lysates from asynchronously growing cells, a 13x-Myc tagged Whi5 protein migrated as a doublet (Fig. 2.2, A). However, in cells arrested in G1 phase by mating pheromone, where CDK activity is inhibited by Far1 (Jeoung et al., 1998; Tyers and Futcher, 1993), Whi5 migrated as a single, faster migrating
FIG. 2.2. Whi5 is phosphorylated throughout the cell cycle.

(A) Cultures of strains expressing Whi5-13xMyc from the endogenous locus were arrested in G1 phase with alpha factor or in metaphase with nocodazole. Western blot of Whi5-13xMyc shows two different migrating forms, indicated by arrows. (B) Western blot of immunopurified Whi5-13xMyc treated with or without lambda phosphatase or protein phosphatase 1 (PP1). Three different migrating forms indicated by arrows represent unphosphorylated, hypo-phosphorylated, and hyper-phosphorylated Whi5. (C) Whi5 immunopurified from asynchronous, alpha factor, or nocodazole arrested cells, treated with lambda phosphatase as in B.
band. In contrast, in cells arrested in M phase by nocodazole, where CDK activity is high, the slower migrating species was enriched. Thus, there are different forms of Whi5 that appear at different cell cycle stages.

To test whether the difference in migration of the Whi5 bands was due to phosphorylation, immunoprecipitates of Whi5 from asynchronous cells were treated with lambda phosphatase or protein phosphatase 1 (Fig. 2.2, B). Upon phosphatase treatment, both the slower and faster migrating species of Whi5 collapsed into a third, fastest migrating un-phosphorylated band. The change in migration of the bands after phosphatase treatment indicates the difference in migration is due to Whi5 phosphorylation.

Next, we compared untreated and lambda phosphatase treated samples of Whi5-13xMyc immunopurified from cells at different stages of the cell cycle. Again, samples of Whi5 immunopurified from asynchronously dividing, mating pheromone arrested, or nocodazole arrested cells were analyzed. Phosphatase treatment of Whi5 from both mating pheromone and nocodazole-arrested cells induced appearance of the fastest migrating band (Fig. 2.2, C). Thus, Whi5 can be separated into three species: the slowest migrating form of Whi5, designated hyper-phosphorylated; the middle form, designated hypo-phosphorylated; and the fastest form, designated un-phosphorylated. We note that the un-phosphorylated form of Whi5 was not found at any cell cycle stage or even in mating pheromone arrested cells. In addition, we observe that Whi5 from mating pheromone arrested cells with inhibited CDK activity shifted downward after phosphatase treatment,
suggesting Whi5 is phosphorylated in early G1 and implicating the presence of non-CDK phosphorylation events.

**Isoelectric Focusing/2D gel analysis of Whi5**

As another measure to confirm the status of Whi5 phosphorylation at different cell cycle stages, isoelectric focusing-2D gel analysis of purified Whi5 was performed. Whi5-13xMyc has a predicted isoelectric point of 4.62, that becomes more acidic with addition of negatively charged phosphate groups. With up to 18 putative phosphorylation sites, the predicted isoelectric point of phosphorylated Whi5-13xMyc is 4.29 (ScanSite, MIT). Samples of immunopurified Whi5-13xMyc from asynchronous, mating pheromone arrested, and nocodazole arrested cells were subject to IEF-2D gels. Additionally, an immunopurified sample from asynchronous cells treated with lambda phosphatase was also included (Fig. 2.3, A). The cell cycle profiles of samples used in this experiment are shown in Fig. 2.3, B.

When separated on IEF strips with a pH gradient of 10 to 3, Whi5 from asynchronous cells is present in two major groups, a group of spots is present in the middle of the strip, with another group of spots situated closer to the acidic end. The large spot seen on the very end of the strip is thought to be precipitated protein that did not separate on the strip. Whi5 purified from mating pheromone arrested cells is present in a group of spots in the middle of the strip. Sample from nocodazole arrested cells is present in a group of spots in the middle of the strip, as well as another group of spots closer to the acidic
FIG. 2.3. IEF-2D Gel Mapping of Whi5.
(A) IEF-2D gel analysis of Whi5-Myc. Anti-Myc Western blots after 2D separation are aligned with basic end, pH 10, and acidic end, pH 3 of IEF strip indicated. Groups of spots representing un-phosphorylated, hypo-phosphorylated, and hyper-phosphorylated Whi5 are indicated. (B) Cell cycle profiles for cell samples used in A and FIG 2.2 A and C.
end. The phosphatase treated sample gives rise to a third group of spots, situated close to the basic end of the strip. Based on these data, in combination with the results showing differential migration in SDS-PAGE in Fig. 2.2, we can designate the group of spots near the basic end as un-phosphorylated; the spots in the middle as hypo-phosphorylated, and the spots near the acidic end as hyper-phosphorylated Whi5. These IEF-2D gels confirm that Whi5 is differentially phosphorylated at different cell cycle stages. Surprisingly, Whi5 in the un-phosphorylated state is not detected.

**Phosphorylation of Whi5 CDK and non-CDK site mutants**

The phosphorylation status of Whi5 mutants was also analyzed by phosphatase treatment. Mutation of the 6 non-CDK sites (A-F) to alanine did not effect the migration of Whi5 in SDS-PAGE compared to wild type Whi5; both hypo- and hyper-phosphorylated bands were apparent in untreated samples, and the un-phosphorylated band was present in phosphatase treated samples (Fig. 2.4, A). Consistent with the mass spec analysis, mutation of Whi5 CDK sites 1-12 to alanine resulted in a single hypo-phosphorylated band that increased mobility after phosphatase treatment. We can conclude that the hyper-phosphorylated band only occurs when CDK sites are left intact. Since both Whi5-6Ala and Whi5-12Ala were found phosphorylated, the non-CDK sites A-F are not essential for CDK site phosphorylation, and *vice versa*. Finally, when all 12 CDK and 6 non-CDK sites were mutated to alanine in Whi5-18Ala, migration did not change upon
FIG. 2.4. Analysis of Whi5 phosphorylation mutants.
(A) Strains with CEN plasmids of phosphorylation mutants of Whi5-13xMyc under the GAL1 promoter were grown in galactose before immunopurification. Whi5-WT, non CDK mutant 6Ala, CDK mutant 12Ala, and CDK/non-CDK mutant 18Ala were treated with phosphatase. Arrows indicate three different phosphorylation forms. (B) Whi5-7Ala CDK mutant expressed from the MET3 promoter immunopurified and treated with phosphatase. (C) Whi5-HA tagged proteins immunopurified and treated with phosphatase.
phosphatase treatment. This result indicates that no phosphate groups that affect migration in a gel were present on the Whi5-18Ala mutant.

We also examined phosphorylation status of another Whi5 CDK mutant. In the study of de Bruin et al, a 7 alanine CDK mutant of Whi5 was generated with CDK sites 2, 4, 5, 8, 9, 10, and 12 mutated to alanine residues (de Bruin et al., 2004). The Whi5-7Ala mutant was expressed under the control of the inducible MET3 promoter. In the presence of methionine, the promoter was repressed, while in the absence of methionine, the promoter was activated. Protein levels of Whi5-WT-13xMyc or Whi5-7Ala-13xMyc CDK mutant from the MET3 promoter were equivalent to protein levels of Whi5-WT-13xMyc expressed from the endogenous WHI5 locus (Fig. 2.4, B). Immunopurified Whi5-7Ala migrated as a single hypo-phosphorylated band, and then shifted to the un-phosphorylated form upon phosphatase treatment (Fig. 2.4, B). This indicates that mutation of 7 CDK sites of Whi5, leaving 5 CDK sites wild type, results in inability to be hyper-phosphorylated but is still sufficient for hypo-phosphorylation.

As a control, we examined phosphorylation of HA tagged Whi5 to ensure the differences in migration in a gel are indeed due to phosphorylation of Whi5 and are not influenced by the 13xMyc tag. Whi5-WT with a 3xHA tag on its C-terminus migrated as a doublet in SDS-PAGE, and shifted to a single faster migrating band upon phosphatase treatment (Fig. 2.4, C). Additionally, Whi5-12Ala CDK mutant with a 3xHA tag migrated as a single, hypo-phosphorylated band when untreated and a faster migrating, hyper-
phosphorylated band after phosphatase treatment (Fig. 2.4, C). Since the same pattern of differential migration is seen in the HA tagged proteins as the Myc tagged versions, we conclude the epitope tag is not influencing Whi5 protein migration.

**32P-ortho-phosphate labeling of Whi5 phosphorylation mutants**

In addition to analysis of Whi5 phosphorylation mutants with phosphatase treatment and electrophoresis, *in vivo* orthophosphate labeling was also performed to verify their phosphorylation status. Cells were labeled with $^{32}$P-ortho-phosphate, and Whi5-3xHA was purified with a double immunoprecipitation to minimize background signal. As expected, wild type Whi5 had the highest amount of radioactive phosphate labeling (Fig. 2.5, A and B). Both Whi5-6Ala and Whi5-12Ala are phosphorylated, although to a lesser extent than wild type Whi5, verifying that the non-CDK or CDK mutants of Whi5 are still phosphorylated. Finally, radioactive signal from Whi5-18Ala was similar to background levels, demonstrating phosphorylation of Whi5-18Ala was negligible. This indicates that beyond the 12 CDK and 6 non-CDK sites, there are no other major phosphorylation sites in Whi5.
FIG. 2.5. Orthophosphate labeling of Whi5 phosphorylation mutants. (A) *In vivo* orthophosphate labeling of Whi5 mutants. Whi5-HA was double immunoprecipitated from cells metabolically labeled with $^{32}$P-phosphate before SDS-PAGE and transfer. Nitrocellulose was exposed for 24 hours before immunoblotting for Whi5-HA. (B) Graph quantifying radioactive counts of Phosphor Image in A.

This experiment was performed in part by Dr. Steven Dowdy, UCSD.
DISCUSSION

**Whi5 CDK and non-CDK phosphorylation**

Whi5 is a negative regulator of G1 cell cycle progression that is inactivated by CDK phosphorylation at Start (Costanzo et al., 2004; de Bruin et al., 2004). Whi5 contains 12 putative CDK phosphorylation sites. However, it has remained unclear if specific CDK phosphorylation sites on Whi5 are utilized for inactivation or if a critical phosphorylation threshold of randomly distributed CDK sites induces inactivation. Here we utilized mass spectrometry analysis to show that from an asynchronous culture, Whi5 is phosphorylated *in vivo* on 10 CDK sites and 6 novel, non-CDK sites.

This analysis expanded upon a previous mass spectrometry study of Whi5 phosphorylation that identified 5 phosphorylated CDK sites (sites 2, 4, 5, 10 and 12) (de Bruin et al., 2004). We did not detect phosphorylation of CDK sites 1 or 2; however, phosphorylation of CDK site 2 was detected by deBruin et al (de Bruin et al., 2004). The fact that neither of our analyses detected phosphorylation of CDK site 1 does not preclude the possibility that it is indeed phosphorylated but just cannot be detected by our methods, perhaps due to the fact that CDK site 1 is very close to the N-terminus at amino acid 5, making a very small tryptic peptide for subsequent analysis. Nevertheless, our cumulative data suggest that 11 of the 12 CDK sites can be occupied *in vivo*, in addition to the 6 novel non-CDK sites.
**Whi5 phosphorylation throughout the cell cycle**

We assessed cell cycle dependency of Whi5 phosphorylation to determine if phosphorylation correlated with Whi5 function. We observed that Whi5 is found hypo-phosphorylated at all stages of the cell cycle, including early G1 in mating pheromone arrested cells. In mitosis, when CDK activity is high, we observed that Whi5 becomes hyper-phosphorylated as well. We did not observe un-phosphorylated Whi5 in vivo, even in mating pheromone arrested cells. Because mating pheromone arrested cells have inhibited CDK activity, the phosphorylated sites are likely non-CDK sites. The hypo-phosphorylation of Whi5 observed in cells in early G1 mirror the hypo-phosphorylated state of the retinoblastoma protein Rb in early G1 in mammalian cells (Ezhevsky et al., 1997).

Isoelectric focusing followed by 2D gel analysis of Whi5 confirmed that different phosphorylated forms of Whi5 are present in mating pheromone arrested cells compared to nocodazole arrested cells. IEF-2D gels also confirm that un-phosphorylated Whi5 is not found in vivo and is only present after phosphatase treatment. This demonstrates that Whi5 is found phosphorylated at all cell cycle stages, and the pattern of phosphorylation changes throughout the cell cycle. Whi5 is hyper-phosphorylated in mitosis, a stage of the cell cycle where it is localized to the cytoplasm and does not bind promoters. Heavy CDK phosphorylation of Whi5 correlates with its inactive state. Conversely, in early G1 when Whi5 is actively repressing promoters, its phosphorylation state is intermediate.
**Phosphorylation status of Whi5 mutants**

The phosphorylation state of Whi5 phosphorylation site mutants was also analyzed to determine how CDK and non-CDK sites contribute to the differently phosphorylated forms of Whi5. Mutating non-CDK sites (6Ala) did not inhibit subsequent phosphorylation of CDK sites, indicating non-CDK phosphorylation is not a prerequisite for CDK phosphorylation. Likewise, mutating CDK sites (12Ala) did not affect non-CDK phosphorylation of Whi5, indicating that CDK and non-CDK phosphorylation events are independent. This observation is supported by the mass spectrometry data, as analysis of Whi5-12Ala showed it retained non-CDK phosphorylation. Mutation of all CDK and known non-CDK sites (18Ala) resulted in a protein that was insensitive to phosphatase treatment, indicating that it was no longer phosphorylated on any novel or cryptic sites.

*In vivo* orthophosphate labeling of Whi5 confirmed the phosphorylation status of the Whi5 mutants. When all CDK and non-CDK sites were mutated to alanine, the Whi5-18Ala mutant had only background levels of incorporated radiolabeled phosphate, confirming that there are no major phosphorylation sites in addition to the 12 CDK and 6 non-CDK sites. The Whi5 CDK mutant and the Whi5 non-CDK mutant were both radiolabeled at intermediate levels, not as high as wild type Whi5, but well over background, confirming they are still phosphorylated *in vivo*. 
Function of Whi5 phosphorylation

Phosphorylation of Whi5 on CDK sites is proposed to contribute to its inactivation and dissociation from promoters (Costanzo et al., 2004; de Bruin et al., 2004). Previous data has shown that expression of the Whi5-7Ala CDK mutant or the Whi5-12Ala CDK mutant does not cause a dramatic cell cycle phenotype (Costanzo et al., 2004; de Bruin et al., 2004). This suggests that Whi5 CDK phosphorylation is not essential for SBF activation and cell cycle progression. Experiments presented in the next chapters will analyze the function of the Whi5-12Ala CDK mutant to determine if CDK sites are necessary for Whi5 function. Regulation of cell size, binding ability to SBF component Swi6, and sub-cellular localization will be analyzed, among others.

Phosphorylation on non-CDK sites is a novel discovery for Whi5. Thus far the function of Whi5 non-CDK phosphorylation is unknown; we have demonstrated that non-CDK phosphorylation is not necessary for subsequent CDK phosphorylation. This eliminates a model wherein non-CDK phosphorylation of Whi5 was required for recognition by CDKs. The function of the non-CDK sites of Whi5 will be analyzed in the next chapters to determine if they are essential for the known function of Whi5, including regulation of cell size and genetic interaction with Swi6.

Chapters 2-6, in part, have been submitted for publication in the journal Eukaryotic Cell. Michelle Wagner, Marcus Smolka, Rob de Bruin, Huilin Zhou, Curt Wittenberg, and Steven Dowdy. The dissertation author was the primary author of this paper.
CHAPTER 3

Functional Analysis of Whi5 Phosphorylation Site Mutants
INTRODUCTION

Whi5 is a phospho-protein that is phosphorylated on at least 10 CDK sites and 6 non-CDK sites *in vivo*. Additionally, Whi5 is not found un-phosphorylated in vivo, suggesting that Whi5 is phosphorylated soon after translation. While the function of novel non-CDK sites has not been investigated thus far, the function of Whi5 CDK sites serves to aid in its inactivation and nuclear export (Costanzo et al., 2004; de Bruin et al., 2004). A C-terminal Whi5 CDK mutant was observed to be nuclear throughout the cell cycle (Costanzo et al., 2004), demonstrating CDK phosphorylation of Whi5 is required for nuclear export.

However, Whi5 CDK sites are not essential to inactivate Whi5 and activate SBF transcription. It has been shown that Whi5 CDK sites are required for proper timing of Start, as a Whi5-7Ala CDK mutant buds at a larger cell size than Whi5-WT cells, and correspondingly delays transcription of SBF targets (de Bruin et al., 2004). Additionally, expression of Whi5-12Ala CDK mutant is not detrimental to cell growth unless also expressed with the Swi6-CDK mutant (Costanzo et al., 2004). Thus, while it is clear that Whi5 CDK site phosphorylation is functioning to induce nuclear export, its requirement in Whi5 inactivation and induction of SBF transcription at Start is less stringent.

In this section we discuss functional assays designed to assess the ability of Whi5-CDK and non-CDK mutants to perform the functions attributed
to wild type Whi5. We demonstrate that Whi5 phosphorylation mutants can regulate cell size, can bind Swi6, and can interact genetically with other components of the molecular machinery involved in Start. We also investigate the regulation of Whi5 non-CDK phosphorylation and demonstrate that it occurs independently of CDK activity. Understanding of the functional ability of Whi5 phosphorylation mutants will provide insight into the functions of the CDK and non-CDK phosphorylation events.
RESULTS

Cell size analysis of Whi5 CDK phosphorylation mutants

*whi5Δ* cells exhibit a small cell size phenotype (Jorgensen et al., 2002). To assess the functionality of Whi5 phosphorylation mutants, we assayed for their ability to rescue *whi5Δ* small size (Fig. 3.1, A). The cell size distribution of wild type parent strain BY4741 (shown as a red tracing) with a mean cell size distribution of 48.2 fL, is considered normal. However, the size distribution for *whi5Δ* cells (shown as a green tracing) with a mean size of 36.2 fL in this experiment is significantly smaller. Introduction of wild type *WHI5* on a CEN plasmid under its native promoter into *whi5Δ* cells (shown as a blue tracing) rescued the small cell size defect and returned the culture to a mean cell size of 53.2 fL. Expression of *WHI5-12Ala* in *whi5Δ* cells (shown as a black tracing) also rescued the small cell size phenotype, with a mean cell size of 53.3 fL. This shows that Whi5-12Ala functioned as well as wild type Whi5 in its ability to influence cell size. Importantly, expression of Whi5-12Ala from its own promoter did not result in a larger cell size. These observations suggest that CDK phosphorylation of Whi5 is not essential in SBF activation, as loss of Whi5 CDK phosphorylation sites did not delay cell cycle progression that would have lead to a larger cell size. Whi5 CDK sites are not essential for regulation of cell size.

We also analyzed how Whi5-WT and Whi5-12Ala influenced cell size when overexpressed from the *GAL1* promoter. The cell size, measured by
FIG. 3.1 Whi5 CDK sites are not necessary for cell size regulation.

(A) Size analysis of whi5Δ cells with CEN plasmids expressing Whi5-WT or Whi5-12Ala CDK mutant from the WHI5 promoter, a 545bp fragment from sequence directly 5′ of the WHI5 open reading frame. (B) Size measured as FSC-H of whi5Δ cells expressing GAL1-WHI5-WT or 12Ala CDK phosphorylation mutant.
FSC-H, was analyzed for cultures grown in raffinose, when expression from the *GAL1* promoter is not active, and in galactose, when expression from the *GAL1* promoter is robust (Fig. 3.1, B). Both wild type BY4741 and *whi5*Δ cells with empty vector were smaller in galactose than raffinose. However, growth in galactose of wild type or *whi5*Δ cells expressing Whi5-WT or Whi5-12Ala resulted in a larger mean cell size. This demonstrates that overexpression of Whi5-WT results in a larger cell size, likely because excess Whi5 is delaying Start. Whi5-12Ala also promoted a larger cell size, indicating it is equally as potent as Whi5-WT in antagonizing Start.

**Whi5 expression levels from the *WHI5* promoter and the *GAL1* promoter**

In order to demonstrate that different levels of Whi5 protein are produced when *WHI5* is under the control of different promoters, we determined the levels of expression from the *WHI5* promoter compared to the *GAL1* promoter. Immunoblot analysis of whole cell lysates to detect Whi5-Myc generated from the endogenous *WHI5* locus compared to levels from a CEN plasmid containing the 545 base pair region 5’ to the *WHI5* open reading frame showed expression via both methods resulted in similar levels of Whi5 protein (Fig. 3.2). Whi5 protein levels were also compared to levels generated by the induced *GAL1* promoter, which were higher (Fig. 3.2). Immunoblot of the same membrane for Cdc28 showed that similar amounts of protein were loaded in each lane. The 545 bp fragment is functional as a *WHI5* promoter,
FIG. 3.2 Characterization of Whi5 expression levels.
Whi5 expression levels from the endogenous locus compared to plasmids containing Whi5 under the control of the 545bp fragment of the \textit{WHI5} promoter or the induced \textit{GAL1} promoter. Cdc28 levels are used as a loading control.
giving similar expression as the promoter at the endogenous locus. Control from the \textit{GAL1} promoter results in expression above physiologic levels.

\textbf{Cell size analysis of Whi5 non-CDK phosphorylation mutants}

Mutants in the non-CDK phosphorylation sites of Whi5 were also assayed for ability to rescue \textit{whi5Δ} cell size. Whi5-WT or phosphorylation mutants were expressed from the inducible \textit{MET3} promoter, so cells were grown in low levels of methionine to activate expression. In this experiment, parent strain BY4741 (shown as a red tracing) had a mean cell size of 35.3 fL, while \textit{whi5Δ} cells (shown as a green tracing) were a mean size of 31.5 fL (Fig 3.2). Expression of \textit{WHI5} wild type, \textit{WHI5-12Ala} (CDK), \textit{WHI5-6Ala} (non-CDK), or \textit{WHI5-18Ala} (CDK and non-CDK) from the inducible \textit{MET3} promoter was able to rescue the small cell size of \textit{whi5Δ} mutants, with mean cell sizes of 42.1, 43.5, 43.2 and 42.4 fL, respectively (Fig. 3.3). We note that the slightly larger cell size observed is likely due to constitutive expression of Whi5 from the \textit{MET3} promoter (de Bruin et al., 2004), but not overexpression, as expression of Whi5 from \textit{MET3} is close to levels of Whi5 generated from its own promoter (Fig. 2.4, B). These observations indicate that in wild type cells, phosphorylation of Whi5 on CDK sites or non-CDK sites is not essential for regulation of cell size.
FIG. 3.3 Whi5 non-CDK sites are not necessary for cell size regulation. Size analysis of whi5Δ cells expressing Whi5-WT, Whi5-12Ala (CDK mutant), Whi5-6Ala (non CDK mutant), or Whi5-18A (CDK and non-CDK mutant) from the MET3 promoter on a CEN plasmid.
**Whi5 CDK mutant interacts with Swi6**

To inhibit SBF transcription, Whi5 binds Swi6 and Swi4 in early G1 (Costanzo et al., 2004; de Bruin et al., 2004). It has also been shown that interaction of Whi5 and Swi6 is detected throughout the cell cycle, even outside of early G1. We assessed the importance of CDK phosphorylation of Whi5 in its interaction with Swi6. When wild type Whi5-13xMyc is expressed with Swi6-3xHA, an immunoprecipitation with anti-Myc antibody purified Whi5 and Swi6 (Fig. 3.4, A) confirming the previously reported observations (Costanzo et al., 2004; de Bruin et al., 2004). Likewise, immunoprecipitation with anti-HA antibody purified both Swi6 and Whi5. Control strains with either Whi5-13xMyc or Swi6-3xHA showed that the anti-Myc and anti-HA antibodies were specific for the tagged proteins. We next examined Whi5-12Ala-13xMyc CDK mutant for its ability to interact with Swi6-3xHA. An immunoprecipitation with anti-Myc antibody to purify Whi5-12Ala also pulled down Swi6 and an anti-HA immunoprecipitation purified both Swi6 and Whi5-12Ala (Fig. 3.4, B). The ability of Whi5-12Ala to interact in vivo with Swi6 means that CDK phosphorylation is not necessary for Whi5 to bind Swi6.

**Whi5 overexpression in strains compromised for Start**

It has previously been shown that Whi5 overexpression in cln3Δ cells or in swi6Δ results in cell lethality with permanent cell cycle arrest in G1 prior to Start (Costanzo et al., 2004). It was also shown that Whi5 overexpression in swi4Δ or wild-type cells does not affect cell viability (Costanzo et al., 2004).
FIG. 3.4. Whi5 CDK mutant interacts with Swi6.

(A) Co-immunoprecipitation of Whi5-WT and Swi6. Lysates of cells with 13x-Myc tagged Whi5, 3x-HA tagged Swi6, or both tagged proteins were subject to immunoprecipitation with anti-Myc or anti-HA antibodies. Antibody complexes and whole cell lysate (WCL) were run on SDS-PAGE and immunoblotted with anti-Myc or anti-HA antibodies. Asterisk indicates non-specific band detected by anti-HA in WCL. (B) Co-immunoprecipitation of Whi5-WT or Whi5-12Ala with Swi6. Strains with 13x-Myc tagged Whi5 and 3x-HA tagged Swi6 were subject to immunoprecipitation conditions as in A.
To test if the ability of overexpressed Whi5 to cause lethality in \( cln3 \Delta \) or in \( swi6 \Delta \) cells was dependent on CDK phosphorylation of Whi5, we tested the Whi5-12Ala mutant in this assay. Similar to wild type Whi5, overexpression of Whi5-12Ala from the \( GAL1 \) promoter did not affect growth of wild type cells, but caused lethality in \( cln3 \Delta \) cells and \( swi6 \Delta \) cells (Fig 3.5, A). Overexpression of Whi5-WT or 12Ala did not cause lethality in \( bck2 \Delta \) cells. While excess Whi5-WT did not affect viability of \( swi4 \Delta \) cells, Whi5-12Ala caused lethality in \( swi4 \Delta \) cells. This demonstrates that wild type and \( bck2 \Delta \) cells can tolerate overexpressed Whi5-12Ala, while \( cln3 \Delta , swi6 \Delta , \) or \( swi4 \Delta \) cells cannot.

To verify that Whi5-12Ala is causing lethality in \( cln3 \Delta \) or \( swi6 \Delta \) cells due to its overexpression and not some novel characteristic of Whi5-12Ala, the experiment was repeated using the \( MET3 \) promoter. When Whi5-WT or Whi5-12Ala is expressed at physiologic levels from the \( MET3 \) promoter, \( cln3 \Delta \) or \( swi6 \Delta \) cells remain viable (Fig 3.5, B). This confirms that the lethality observed with expression of Whi5-WT and Whi5-12Ala from the \( GAL1 \) promoter is due to the high levels of Whi5 present. It also indicates that there may be some other mechanism utilized by the cell to activate Start, as cells without the cyclin Cln3 and having CDK mutant Whi5-12Ala at physiologic levels are still viable and able to grow in this assay.
FIG. 3.5. Expression of Whi5 CDK mutant in strains compromised for Start.

(A) Wild type, cln3Δ, bck2Δ, swi4Δ, or swi6Δ cells with empty vector or CEN plasmids with Whi5-WT or 12Ala under control of the GAL1 promoter. Cells were spotted in serial five fold dilutions on glucose or galactose media and incubated 48 hours. (B) cln3Δ or swi6Δ cells with Whi5-WT or 12Ala under the control of GAL1 or MET3 promoters. Cells were spotted on galactose media without methionine to induce both the GAL1 and MET3 promoters.
Overexpression of Whi5 phosphorylation mutants in cln3Δ cells

Because overexpression of Whi5-12Ala that caused lethality in cln3Δ cells indicates CDK phosphorylation is not necessary for Whi5 to inhibit Start, we wanted to determine if the non-CDK sites of Whi5 were important in its ability to antagonize cell cycle progression. We utilized the same assay where viability of cln3Δ cells overexpressing Whi5 non-CDK mutants was assessed. GAL1 overexpression of Whi5-6Ala non-CDK mutant, or Whi5-18Ala CDK and non-CDK mutant were able to cause lethality of cln3Δ cells (Fig. 3.6). This result suggests that hypo-phosphorylation of either CDK or non-CDK sites are not required for Whi5 to antagonize Cln3 activity.

Expression of Whi5 phosphorylation mutants in cln3Δbck2Δ cells

In the background of cln3Δ mutant, BCK2 becomes essential (Wijnen and Futcher, 1999). It has previously been shown that cln3Δbck2Δ double knockout cells are inviable and permanently arrest in early G1, unable to activate Start (Wijnen and Futcher, 1999). The work of deBruin et al. shows that Whi5 is an essential target of Cln3 and Bck2, as triple knockout cln3Δbck2Δwhi5Δ cells are viable (de Bruin et al., 2004). To test the ability of Whi5 phosphorylation mutants to inhibit Start, they were expressed in triple knockout cln3Δbck2Δwhi5Δ cells (Fig. 3.7). As previously shown, cln3Δbck2Δ cells kept alive with YCpCLN3::URA3 are viable, but when plated on media
FIG. 3.6. Expression of Whi5 non-CDK mutants in cln3Δ cells. Wild type or cln3Δ cells with CEN plasmids of empty vector, Whi5-WT, 12Ala CDK mutant, 6Ala non-CDK mutant, or 18Ala CDK and non-CDK mutant under control of the GAL1 promoter. Serial five fold dilutions were plated on glucose or galactose and incubated 48 hours.

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**FIG. 3.7. Expression of Whi5 phosphorylation mutants in cln3Δbck2Δ cells.**

cln3Δbck2Δ cells with YCpCLN3::URA3 plasmid plated on media without uracil or on media with uracil and 5’-FOA to select for loss of the plasmid. Also plated are cln3Δbck2Δwhi5Δ triple knockout cells with YCpCLN3::URA3 plasmid and either empty vector or Whi5-WT, 12Ala, 6Ala, or 18Ala under control of the MET3 promoter. The media containing uracil and 5’FOA but lacking methionine selects for loss of the CLN3 plasmid and induces expression of the MET3-WHI5 constructs.
containing 5-FOA to select for loss of the URA3 plasmid, they are inviable. In contrast, when WHI5 is deleted in this background, the cells are viable even with the loss of the URA3 plasmid. Reintroduction of Whi5-WT, expressed from the MET3 promoter, causes lethality in cln3Δbck2Δwhi5Δ cells plated on 5-FOA. Likewise, expression of Whi5-12Ala, Whi5-6Ala, or Whi5-18Ala from the MET3 promoter was able to cause lethality in cln3Δbck2Δwhi5Δ cells plated on 5-FOA (Fig. 3.7). The phosphorylation mutants are equally capable of functioning like Whi5-WT in this assay, indicating phosphorylation of Whi5 is not required for its ability to inhibit Start and cause lethality when Cln3 and Bck2 cannot activate cell cycle progression.

**Phosphorylation of Whi5 non-CDK sites is independent of Cln:CDK activity**

Thus far it has been established that the non-CDK sites of Whi5 are not required for Whi5 CDK phosphorylation, regulation of cell size, or ability of Whi5 to repress transcription at Start. In an attempt to further characterize the non-CDK sites, we assessed whether or not the non-CDK phosphorylations are dependent on Cln1/2/3:Cdc28 cyclin dependant kinase activity. We utilized triple knockout cln1Δcln2Δcln3Δ cells that are inviable (Tyers et al., 1993) but are maintained with a GAL1-CLN3 plasmid to allow growth. These cells were transformed with empty vector, MET3-WHI5-WT-13xMyc, or MET3-WHI5-12Ala-13xMyc. Cells were viable and cycled normally when grown in galactose media, but arrested cell division and accumulated in G1 phase when
switched to glucose media to represses \textit{GAL1-CLN3} (Fig. 3.8, A). We took arrested cells in glucose media and inoculated them into media lacking methionine to induce the \textit{MET3} promoter and Whi5-Myc expression (Fig. 3.8, B). Even when arrested in G1 phase without Cln1, Cln2, or Cln3 activity cells can still effectively activate the \textit{MET3} promoter. We then assayed migration in a gel of Whi5-WT or Whi5-12Ala that was produced in cells grown in glucose that were arrested in G1 without Cln activity. The migration of both Whi5-WT and Whi5-12Ala was sensitive to phosphatase treatment (Fig 3.8, C). Whi5 is still hypo-phosphorylated in cells lacking Cln:Cdc28 kinase activity, indicating that non-CDK phosphorylation occurs independent of Cdc28 activity.

\textbf{Whi5 non-CDK sites are occupied during non-fermentive growth}

One of the 6 non-CDK sites of Whi5, Ser149 designated site C (Fig 2.1), is a near consensus site for cyclic-AMP activated protein kinase (PKA). The published consensus site for PKA is R-(K/R)-x-(S/T) (Ptacek et al., 2005) and Ser149 is preceded by two arginine residues. Non-CDK site C is also in between CDK site 7 and CDK sites 8, 9, and 10; its close proximity to CDK sites prompted us to examine its phosphorylation further. Increased levels of cAMP activate PKA when cells are grown in glucose media (Tokiwa et al., 1994). This has been shown to be important for cell size regulation, as cells delay entry into the cell cycle in order to reach a larger cell size in rich media (Flick et al., 1998; Rupes, 2002). We hypothesized that Whi5 non-CDK sites may be phosphorylated in response to glucose signaling. All assays on Whi5
FIG. 3.8. Phosphorylation of Whi5 non-CDK sites is CDK-independent. (A) Cell cycle profiles of \( \Delta \Delta \Delta \) GAL1-CLN3 cells with indicated plasmids. Cells arrest in G1 phase upon growth in glucose. (B) Western blot for Whi5-Myc induced in cells from A grown without methionine. (C) Whi5-Myc was immunopurified from cells in A grown without methionine and treated with lambda phosphatase.
phosphorylation thus far had been performed in glucose or galactose media, both of which are fermentable sugars. To see if phosphorylation of Whi5 non-CDK sites were dependent on glucose signaling, cells were grown in glycerol/ethanol media where glucose signaling would be repressed. When asynchronously grown in the non-fermentable carbon source, Whi5-WT was still sensitive to change in migration upon phosphatase treatment (Fig. 3.9). Additionally, upon arrest with mating pheromone, Whi5-WT changed migration after phosphatase treatment. This indicates non-CDK phosphorylation was still occurring in cells grown without glucose. This does not completely rule out the possibility that PKA targets Whi5, and the other non-CDK sites are occupied in non-fermenting conditions to cause the sensitivity to phosphatase. Thus far we have not been able to attribute any function to the non-CDK sites of Whi5.
FIG. 3.9. Whi5 non-CDK sites are occupied during non-fermentive growth.
Western blot of Whi5-Myc immunopurified from wild type cells grown in 2% glycerol 1% ethanol media and phosphatase treated.
DISCUSSION

Cell size regulation by Whi5 phosphorylation mutants

In yeast cells must reach a critical cell size before entry into the cell cycle (Johnston et al., 1979; Johnston et al., 1977). Whi5 inhibits passage through Start until cells reach their critical cell size and signal for cell cycle entry. Cells without WHI5 are small in size because they lack the Whi5 inhibitory function that normally delays Start until the critical size is achieved. Thus, whi5Δ cells enter the cell cycle prematurely. To test the function of Whi5 phosphorylation sites in control of cell size, Whi5 phosphorylation mutants were assayed for the ability to rescue the whi5Δ small cell size. The 12Ala CDK mutant, 6Ala non-CDK mutant, and 18Ala double mutant were all able to promote a wild type cell size with equal efficiency as Whi5-WT.

Phosphorylation of Whi5 is thus not necessary for regulation of cell size. This has multiple implications. First, phosphorylation of Whi5 on CDK or non-CDK sites is not required for Whi5 to inhibit Start, because cells expressing Whi5 phosphorylation mutants were not small, indicating timing of passage through Start occurred at the proper size. Second, phosphorylation of Whi5 on CDK or non-CDK sites is not required to inactivate Whi5 for SBF activation. Cells expressing Whi5 phosphorylation mutants were not larger in size, indicating there was not a delay in SBF activation when Whi5 phosphorylation is eliminated.
Our results show that the timing of cell cycle entry is not significantly affected by Whi5 phosphorylation site mutations. Our results differ from those of de Bruin et al, who report that Whi5-7Ala CDK mutant expressed from the MET3 promoter caused a measurable defect in cell cycle progression, with cells inducing maximum SBF transcription at a ~10% larger cell size (de Bruin et al., 2004). The difference in results may be due to strain background differences or the use of the heterologous promoter. Additionally, plasmids with Whi5 driven by the WHI5 promoter or the MET3 promoter were used in this study, while deBruin et al utilized a MET3 driven Whi5 integrated at the URA3 locus; the different methods of expression could also be a potential source of the variation in the data. Nevertheless, the reported effect of Whi5-7Ala was not as significant as would be predicted if Whi5 phosphorylation was essential for SBF activation and timing of Start.

**Whi5-12Ala physical interaction with Swi6**

It has been reported that the retinoblastoma protein (Rb) is hypo-phosphorylated on CDK sites when it is found binding and repressing E2F transcription factors at E2F target promoters (Ezhevsky et al., 1997). It is currently being studied whether or not the hypo-phosphorylation of Rb is required for binding E2F and transcriptional repression activity (G. Shapiro, S.F. Dowdy, unpublished observations). We tested if Whi5-12Ala could interact with Swi6, a member of the SBF transcriptional complex. We were able to show Whi5-12Ala CDK mutant interacts with Swi6 just as well as Whi5-
WT. This demonstrates that Whi5 CDK sites are not required for Whi5 binding Swi6, and support the cell size data demonstrating Whi5 CDK sites are not necessary for Whi5 to inhibit Start.

**Whi5 phosphorylation mutants retain ability to inhibit Start**

It was previously shown that overexpression of Whi5-WT in cells compromised for Start, such as *cln3Δ* or *swi6Δ*, caused cell lethality. Overabundance of a repressor of Start (Whi5) in strains already impaired in their ability to activate SBF transcription creates a situation where passage through Start is not possible, causing a permanent G1 arrest. We tested Whi5 CDK mutants and non-CDK mutants for their ability to inhibit Start when overexpressed in cells with deletions of genes involved in positive regulation of Start. All phosphorylation mutants of Whi5 tested were able to cause lethality similar to Whi5-WT, indicating phosphorylation is not required for Whi5 inhibitory activity when overexpressed.

We also assessed the viability of cells expressing the Whi5-12Ala phosphorylation mutant from the *MET3* promoter at physiologic levels. It did not cause lethality in *cln3Δ* or *swi6Δ* cells, indicating that physiologic expression of Whi5-12Ala in strains compromised for Start is not sufficient to cause lethality. This shows that the lethality seen in the experiments described above is due to Whi5 overexpression, and also shows that having the Whi5-12Ala CDK mutant in cells does not cause a dramatic phenotype, even in cells already impaired in their ability to enter the cell cycle.
Another analysis expressing Whi5 phosphorylation mutants at physiologic levels was performed in \( \text{cln3}\Delta bck2\Delta \text{whi5}\Delta \) cells to determine if any phosphorylation of Whi5 was required when Start was severely compromised. Bck2 is an activator of Start and functions independently of CDK activity. Its function is not fully understood, but it becomes essential in \( \text{cln3}\Delta \) cells, meaning \( \text{cln3}\Delta bck2\Delta \) double knockout cells are lethal (Wijnen and Futcher, 1999). The study of de Bruin et al showed that Whi5 is an essential target of Cln3/Bck2 because deletion of WHI5 in \( \text{cln3}\Delta bck2\Delta \) cells allows for cell viability (de Bruin et al., 2004). Introduction of physiologic levels of Whi5-WT or Whi5 phosphorylation mutants into \( \text{cln3}\Delta bck2\Delta \text{whi5}\Delta \) cells resulted in cell lethality; thus, Whi5 phosphorylation is not required for its repressive activity, as phosphorylation mutants are equally as functional as Whi5-WT in their ability to inhibit Start in this assay. In addition to showing that phosphorylation is neither essential for Whi5 inactivation nor passage through Start with cell size experiments, we have demonstrated that Whi5 hypo-phosphorylation is not essential for inhibitory function.

**Origin of non-CDK phosphorylation**

The non-CDK sites are not essential for the repressive activity of Whi5, its ability to be inactivated, or for subsequent CDK phosphorylation. Further investigation is needed to explore the biological function of the non-CDK sites. Determining the kinase(s) that target the non-CDK sites could give insight into their function. We demonstrated that the Whi5 is hypo-
phosphorylated in cells without Cln1/2/3 activity in G1, meaning that the phosphorylation of non-CDK sites does not depend on Cln:Cdc28 activity. We also showed that Whi5 is hypo-phosphorylated in mating pheromone arrested cells grown in glycerol/ethanol media, suggesting that the bulk of non-CDK phosphorylation does not result from glucose signaling. Further experiments could be performed to determine if Whi5 has a role in transcriptional repression to lengthen early G1 in glucose media, studying the putative PKA phosphorylation site Ser 149 in Whi5. We note that five out of 6 of the non-CDK sites are followed by acidic residues and conform to the consensus sequences for casein kinase I or casein kinase II (CKA1/2) (Ptacek et al., 2005), that is known to target transcription factors and RNA polymerases (Glover, 1998). Thus, the non-CDK phosphorylation may be a result of Whi5 binding to the SBF transcription factor complex. However, we found that non-CDK sites do not dramatically affect regulation of Whi5 with respect to cell size and timing of Start. Additional investigation is needed to determine the biological function of Whi5 non-CDK phosphorylation and identify the kinase(s) responsible.

Chapters 2-6, in part, have been submitted for publication in the journal Eukaryotic Cell. Michelle Wagner, Marcus Smolka, Rob de Bruin, Huilin Zhou, Curt Wittenberg, and Steven Dowdy. The dissertation author was the primary author of this paper.
CHAPTER 4

Redundant phosphorylation of Whi5 and Swi6
INTRODUCTION

When expressed in an otherwise wild type cell, the 12Ala CDK mutant of Whi5 does not cause a dramatic cell cycle phenotype. Whi5-12Ala expression from the WHI5 promoter, the MET3 promoter, or the GAL1 promoter is able to rescue the small cell size of whi5Δ cells equally as well as wild type Whi5. The CDK sites of Whi5 are therefore not necessary for the proper coordination of cell size with cell cycle entry. Whi5 is a dose dependent cell cycle inhibitor, but does not require phosphorylation to perform that role. In cln3Δ cells, overexpression of Whi5-12Ala is equally able to cause permanent G1 arrest and cell lethality as Whi5-WT. Likewise, expression of Whi5-WT or Whi5-12Ala in triple knockout cln3Δbck2Δwhi5Δ cells caused cell cycle arrest and lethality. Thus, unlike the mechanism proposed for Rb regulation, Whi5 hypo-phosphorylation on CDK sites is not required for its transcriptional repressive function.

In addition to CDK phosphorylation, we have demonstrated that non-CDK phosphorylation of Whi5 is similarly unnecessary for Whi5 regulation. Whi5 non-CDK mutants were able to rescue the small cell size of whi5Δ cells, indicating non-CDK sites are also unnecessary for regulation of cell size. They are similarly not required for Whi5 transcriptional repressive function as well. Whi5 non-CDK mutants are able to cause lethality when overexpressed in cln3Δ cells and also when expressed in cln3Δbck2Δwhi5Δ cells. We
additionally showed that non-CDK phosphorylation is independent of CDK activity.

While function of the non-CDK sites of Whi5 has not been assigned, in this section we present data demonstrating that Whi5 CDK sites are necessary for function, and in fact cell viability, when Whi5-12Ala is overexpressed in Swi6 CDK mutant cells. We perform a mutational analysis of Whi5 CDK sites and define the necessary phosphorylation sites as four C-terminal CDK targets. This mutational analysis proves that Whi5 inactivation relies on 4 specific CDK sites. In an attempt to understand how and why the Whi5-12Ala Swi6-SA4 cells are arresting and unable to divide, we perform cell cycle analysis on Swi6-SA4 and show that it functions normally with respect to regulation of cell size and ability to properly regulate SBF and MBF target gene expression. We also analyzed the effect of overexpression of Whi5-12Ala. Overall we confirm that phosphorylation of Whi5 and Swi6 is redundant, and depends on 4 specific CDK sites of Whi5.
RESULTS

**Whi5 CDK mutant in combination with Swi6 CDK mutant causes lethality**

Expression of various Whi5 CDK phosphorylation mutants in wild type cells does not result in a dramatic phenotype (Costanzo et al., 2004; de Bruin et al., 2004). However, overexpression of Whi5-12Ala in cells that harbor the Swi6-SA4 mutant lacking four CDK sites results in a severe growth defect (Costanzo et al., 2004), suggesting that phosphorylation of either Whi5 or Swi6 is required for SBF activation and cell cycle promotion. We repeated this experiment in our parent strain background. In swi6Δ cells containing YEpSWI6-WT, overexpression of Whi5-WT or Whi5-12Ala from the GAL1 promoter does not cause any growth defect in comparison to cells with empty vector (Fig. 4.1). Likewise, swi6Δ cells containing YEpSWI6-SA4 and overexpressed Whi5-WT are viable. In contrast, swi6Δ YEpSWI6-SA4 cells with overexpressed Whi5-WT have a severe growth defect and do not form colonies in this assay. This corroborates the previous reports that in combination, cells with Whi-12Ala and Swi6-SA4 do not divide. When examined microscopically, Whi5-12Ala Swi6-SA4 cells are large, elongated, and unbudded, indicating that they are likely arresting permanently in early G1 before Start.
FIG. 4.1. Whi5 CDK mutant causes lethality with Swi6 CDK mutant.
Cells with wild type Swi6 or phosphorylation mutant Swi6-SA4 with Whi5-WT or 12Ala under control of the GAL1 promoter. Serial five fold dilutions were plated on glucose or galactose and incubated 48 hours.
**Whi5 non-CDK mutant is viable with Swi6 CDK mutant**

We next tested whether the non-CDK sites of Whi5 are essential for viability and passage through Start in the presence of Swi6-SA4, similar to CDK sites. Whi5 mutants containing alanine substitutions for the 6 non-CDK sites (Whi5-6Ala), and all CDK and non-CDK sites (Whi5-18Ala) were tested for genetic interaction with Swi6-SA4. In swi6Δ cells expressing wild type Swi6, all of the WHI5 constructs, WHI5-WT, WHI5-12Ala, WHI5-6Ala, or WHI5-18Ala overexpressed from the GAL1 promoter, showed no change in growth rate as detected in this assay (data not shown). However, in combination with SWI6-SA4, WHI5-12Ala and WHI5-18Ala cells were inviable, whereas cells expressing WHI5-WT or WHI5-6Ala were viable (Fig. 4.2). Thus, phosphorylation of Whi5 non-CDK sites is not necessary for SBF activation and passage through Start in a SWI6 or SWI6-SA4 genetic background. We are currently unable to ascribe a biological function for phosphorylation of Whi5 at the 6 non-cdk sites.

**Specific CDK phosphorylation sites of Whi5 are required for function**

Although phosphorylation of multiple Whi5 CDK sites was detected in vivo (Fig. 2.1 and de Bruin et al.) (de Bruin et al., 2004), it is not known whether specific CDK sites or a threshold amount of phosphorylation determines Whi5 inactivation and dissociation from SBF. To test these two competing hypotheses of Whi5 inactivation, we performed a mutational analysis of Whi5 CDK sites and assayed for genetic interaction with the SWI6-
FIG. 4.2. Whi5 non-CDK mutant is viable with Swi6 CDK mutant. Cells with SWI6-SA4 and WHI5-WT or phosphorylation mutants under control of the GAL1 promoter. Serial five fold dilutions were plated on glucose or galactose and incubated 48 hours.
SA4 allele (Fig. 4.3). Consistent with previous reports (Costanzo et al., 2004), none of the Whi5 CDK mutants assayed caused growth defects when induced in cells expressing wild type Swi6 (data not shown). Similar to WHI5-12Ala, expression of WHI5-7Ala (CDK sites 2, 3, 5, 8, 9, 10, and 12 mutated to alanine) (de Bruin et al., 2004) caused lethality in combination with SWI6-SA4 (Fig. 4.3, lines 2 & 3).

Based on a preliminary survey of phosphorylation site mutants, we initially focused our attention on the C-terminal CDK phosphorylation sites. Four C-terminal CDK sites of Whi5 (8, 9, 10, and 12) as a group were necessary and sufficient for viability with Swi6-SA4. When WHI5-4Ala, with the four sites mutated to alanine, is expressed in combination with SWI6-SA4, cells were inviable (Fig. 4.3, line 4). In contrast, when WHI5-8Ala\(^1\), where the other 8 CDK sites in Whi5 are mutated to alanine, was expressed with SWI6-SA4, cells were viable (Fig. 4.3, line 5). This demonstrates that CDK sites 8, 9, 10, and 12 are specifically required for Whi5 inactivation in this assay. These four CDK sites are marked with a star in the schematic representation of the Whi5 protein (Fig. 4.3, bottom). To rule out the possibility that phosphorylation of any four CDK sites was sufficient to inactivate Whi5, several constructs having four or more wild type CDK sites were also analyzed. For example, when four N-terminal CDK sites (2, 3, 4, and 5) were wild type while the other eight CDK sites were alanine in WHI5-8Ala\(^2\) (Fig. 4.3, line 6), the SWI6-SA4 cells were still inviable, suggesting that the four N-terminal sites were not sufficient to allow for viability.
FIG. 4.3. Mutational analysis of Whi5 CDK sites with Swi6 CDK mutant. (A) Expression of Whi5-WT or various CDK phosphorylation mutants from the GAL1 promoter in swi6Δ [SWI6-SA4] cells. Five fold serial dilutions of cells were plated on galactose media and incubated for 48 hours. (B) Schematic representation of the Whi5 protein. Triangles indicate CDK phosphorylation sites. Sites 8, 9, 10, and 12 are marked with an asterisk.
Additional analysis of CDK sites 8, 9, 10, and 12 was performed to assess their function as single sites, pairs of sites, or in groups of three. Mutation of site 10 or 12 alone, or pairs of sites (10 and 12; or 8 and 9) to alanine, while leaving the other CDK sites wild type did not affect cell growth (Fig. 4.3, lines 7-10). This demonstrates that no single C-terminal CDK site or pair of sites was necessary for viability. Conversely, mutating all other CDK sites to alanine, leaving only single CDK sites 10 or 12, or pairs of sites (10 and 12; or 8 and 9) wild type did not permit cell growth (Fig. 4.3, lines 11-14). This indicates that phosphorylation of a single site or pair of sites is not sufficient to support cell viability. Interestingly, when three CDK sites (8, 9, and 12; or 8, 9, and 10) were left wild type, they were sufficient to allow growth (Fig. 4.3, line 15), indicating 3 out of the 4 C-terminal CDK sites are sufficient for viability.

However, Whi5 regulation by phosphorylation is complex in that some combinations of C-terminal mutations with other CDK sites influenced viability. For example, mutating 3 of the 4 C-terminal sites to alanine in Whi5-3A\(^1\) (sites 8, 9, and 12) still allows for cell viability, while having those three sites mutated to alanine in combination with 3 N-terminal CDK site mutations in Whi5-6A\(^1\) results in cell lethality (Fig. 4.3, lines 17 & 18). When only CDK site 10 is left wild type, as it was in Whi5-11A\(^2\) (Fig. 4.3, line 12), it was not sufficient to support cell viability. So in Whi5-3A\(^1\), where CDK site 10 was the only C-terminal site left wild type, it must be functioning in collaboration with other N-terminal sites to allow for cell growth.
A similar complex regulation is seen for CDK site 12 as well. In mutant Whi5-3A² sites 8, 9, and 10 are mutated to alanine, leaving site 12 wild type and cells are viable (Fig. 4.3, line 19). Additionally, in mutant Whi5-6A² sites 8, 9, 10 are mutated to alanine in addition to 3 N-terminal CDK site mutations and cells remain viable (Fig. 4.3, line 20). However, when only CDK site 12 is left wild type, in Whi5-11A¹, cells did not grow (Fig. 4.3, line 11). CDK site 12 is not sufficient on its own to support viability, but in combination with some N-terminal sites left intact cells are alive. Further investigation is needed to fully understand the contribution of N-terminal CDK sites to Whi5 regulation.

**Functional Analysis of Swi6-SA4 in cell size**

In order to better understand how Whi5-12Ala is causing lethality in combination with Swi6-SA4 we sought to investigate how Swi6-SA4 functions in comparison to Swi6-WT. We first assayed for the ability of Swi6-SA4 to rescue the large cell size of swi6Δ cells. In comparison to the size distribution of wild type cells (shown as a red tracing) swi6Δ cells (shown as a green tracing) were larger in size (Fig 4.4 A and B). SWI6-WT on an episomal plasmid under control of its own promoter is able to rescue the large cell size of swi6Δ cells (shown as a blue tracing). Similarly, SWI6-SA4 is also able to rescue the large cell size (shown as a black tracing) and returns the size distribution closer to that of wild type cells. The slightly smaller mean cell volume of swi6Δ cells with either SWI6-WT or SWI-SA4 is likely due to the
FIG. 4.4. Swi6-SA4 rescues the large size phenotype of swi6Δ cells.  
(A) Cell size distribution of wild type cells (BY4741) compared to swi6Δ cells and swi6Δ cells containing 2µ plasmids of SWI6-WT or SWI6-SA4.  
(B) Graph of mean cell size of samples in A.
higher copy number per cell of episomal plasmids. Nevertheless, Swi6-SA4 is equally functional to Swi6-WT in regulating cell size.

**Functional Analysis of Swi6-SA4 in regulation of transcription**

We also assessed ability of Swi6-SA4 to regulate SBF and MBF transcription in comparison to Swi6-WT. swi6Δ cells expressing either Swi6-WT or Swi6-SA4 were synchronized with mating pheromone in early G1. Both cultures had less than 10% budded cells (Fig. 4.5, A). The cells were released from mating pheromone arrest and followed as they progressed through the cell cycle. Samples taken every 10 minutes to assess budding index showed that the two cultures were budding at similar rates (Fig. 4.5, A). They both began budding between the 30 and 40 minute time points. RNA was harvested from samples taken from this experiment and assessed for SBF and MBF transcript levels by real time reverse transcriptase PCR. Transcript levels of SBF target gene *CLN2* were low at 10 minutes after pheromone release and reached a maximum peak at 20 minutes in both cultures (Fig. 4.5, B). Transcript levels of *CLN2* began to decline from 30 minutes onward in both samples.

Transcript levels of another SBF target gene, *SVS1* were also examined (Fig. 4.5, C). Similar to *CLN2*, *SVS1* transcript levels began to increase 20 minutes after mating pheromone release in both Swi6-WT and Swi6-SA4 samples. *SVS1* transcript reached its peak at 30 minutes and began to decline thereafter with similar kinetics in both samples. Regulation of
FIG. 4.5. Induction of SBF and MBF target genes in SWI6-SA4 cells. (A) Budding index of swi6Δ cells containing 2µ plasmids with SWI6-WT or SWI6-SA4 synchronized with alpha factor and released. Samples were analyzed every 10 minutes. SWI6-WT is represented in blue (diamonds), SWI6-SA4 in black (squares). (B) CLN2 (C) SVS1 and (D) RNR1 transcript levels from synchronized cells in A. RNA levels determined by Real Time RT-PCR are presented as a fold induction relative to the lowest level observed in both SWI6-WT and SWI6-SA4 time courses after normalization of all values to the ACT1 RNA level in the same samples.
SBF transcription after mating pheromone arrest and release was normal in Swi6-SA4 cells in comparison to Swi6-WT. We also analyzed transcript levels of *RNR1*, an MBF target. For both Swi6-WT and Swi6-SA4 samples, *RNR1* transcript was induced at the 20 minute time point and declines at later time points with similar kinetics (Fig. 4.5, D). We therefore conclude that Swi6-SA4 is able to function normally, similar to Swi6-WT, in regulation of SBF and MBF transcription from early G1, past Start, and as cells enter late G1 and S phase.

**Overexpression of Whi5 or Whi5-12Ala causes a G1 delay**

Thus far we have demonstrated that overexpression of Whi5-12Ala, but not Whi5-WT, caused lethality in cells also expressing Swi6-SA4. Analysis of the SWI6-SA4 allele showed that regulation of transcription was similar to SWI6-WT cells. In order to investigate the nature of the lethality resulting from Whi5-12Ala Swi6-SA4 expression, we next analyzed the effect of overexpression of Whi5. When Whi5-WT was induced from the GAL1 promoter in wild type cells, it caused a cell cycle delay and accumulation of cells in G1 (Fig. 4.6, A, blue bars). When Whi5-WT expression was repressed by growth in glucose media, cell cycle distribution was typical of an asynchronously dividing culture with greater than 60% of cells in S/G2/M phases of the cell cycle. In contrast, induction of Whi5-WT by growth in galactose resulted in a culture with cells accumulated in G1, with only 25% of cells in S/G2/M. A similar result was observed with overexpression of Whi5-12Ala in wild type cells. Repression of expression of Whi5-12Ala resulted in
FIG. 4.6. Overexpression of Whi5 causes a G1 delay in wild type and SWI6-SA4 cells.

(A) Budding index of BY4741 wild type cells (blue) or swi6Δ [SWI6-SA4] cells (black) with empty vector, WHI5-WT, or WHI5-12Ala under the GAL1 promoter grown in glucose or galactose. (B) Wild type cells or swi6Δ [SWI6-SA4] cells with empty vector, WHI5-WT, or WHI5-12Ala under the GAL1 promoter were synchronized with alpha factor in raffinose media then galactose was added to the cultures to induce the GAL1 promoter. Cells were then released into galactose media and budding index measured every 15 minutes.
an asynchronous culture with greater than 60% of cells in S/G2/M phases. However, induction of Whi5-12Ala also induced a G1 accumulation of cells, reducing the number of cells in S/G2/M to only 30% (Fig 4.6, A, blue bars).

Overexpression of Whi5-WT or Whi5-12Ala both induced a G1 accumulation of cells in a wild type background. We also analyzed the effect of Whi5 overexpression in cells expressing Swi6-SA4. Overexpression of Whi5-WT in Swi6-SA4 cells caused a G1 delay, with only 29% of cells in S/G2/M phases, in contrast to Swi6-SA4 cells where Whi5-WT was repressed and greater than 70% of cells are in S/G2/M (Fig. 4.6, A, black bars). Similar results were seen with Whi5-12Ala overexpression in Swi6-SA4 cells. When Whi5-12Ala is repressed in Swi6-SA4 cells, greater than 70% of cells are in S/G2/M phases. In contrast, overexpression of Whi5-12Ala in Swi6-SA4 cells resulted in a G1 delay, with only 24% of cells in S/G2/M. Both Whi5-WT and Whi5-12Ala induce a similar G1 delay in Swi6-SA4 cells.

While it had been previously shown that overexpression of Whi5-WT causes a G1 delay (Costanzo et al., 2004), this result was surprising. When Whi5 was under the control of the GAL1 promoter and cells were plated on galactose media, Whi5-WT Swi6-SA4 cells were alive while WHI5-12Ala Swi6-SA4 cells did not grow (Fig. 4.1). The fact that these two cultures of cells both accumulated in G1 after 5 hours of growth in liquid culture (Fig. 4.6, A) is in contrast to the result seen on solid media. When liquid cultures of Swi6-SA4 cells overexpressing either Whi5-WT or Whi5-12Ala were examined at a later time point after 24 hours of growth in galactose, the results correlated with
those seen with growth on solid media. Swi6-SA4 cells with empty vector or \textit{GAL1-WHI5-WT} both had budding indexes of 30% and 32% after 24 hours of growth in galactose. In contrast, Swi6-SA4 cells with \textit{GAL1-WHI5-12Ala} had a budding index of only 7% after 24 hours of growth in galactose. In addition, Swi6-SA4 Whi5-12Ala cells were un budded, large, and elongated, with cell debris and dead cells present. This indicates that Swi6-SA4 cells with Whi5-WT are able to overcome the G1 delay induced by Whi5 overexpression, while Swi6-SA4 cells with Whi5-12Ala are permanently arrested in G1.

**Overexpression of Whi5 causes a dramatic G1 delay in Swi6-SA4 cells**

While we have demonstrated that Whi5 overexpression causes a G1 delay in wild type and Swi6-SA4 cells when grown in asynchronous cultures, we also wanted to investigate the effect of Whi5 overexpression on synchronized cultures to determine if Whi5-12Ala had a different effect than Whi5-WT in Swi6-SA4 cells. In this experiment, cells were synchronized with mating pheromone in raffinose media, and then galactose was added to induce expression of \textit{GAL1-WHI5}. After incubation of mating pheromone arrested cells with galactose for one hour, cells were washed and released into fresh galactose containing media and budding index was examined every 15 minutes. Wild type cells with empty vector began budding between 30 and 45 minutes, reaching a peak of budded cells at 90 minutes after release (Fig. 4.6, B, blue line). A similar result was seen with Swi6-SA4 cells with empty vector (Fig. 4.6, B, black line). Wild type cells with Whi5-WT or Whi5-12Ala
under control of the \textit{GAL1} promoter both exhibited a delay in budding, starting budding between 60 and 90 minutes (Fig. 4.6, B, purple and light blue lines). Finally, Swi6-SA4 cells with Whi5-WT or Whi5-12Ala under control of the \textit{GAL1} promoter had a dramatic delay in budding, with no significant budding observed during the time course of this experiment. In experiments in liquid media with shorter time courses, both Whi5-WT and Whi5-12Ala overexpression cause a G1 delay in Swi6-SA4 cells. It is only in experiments over a longer course of time where the difference between \textit{GAL1} induced Whi5-WT and Whi5-12Ala in Swi6-SA4 cells is evident.
DISCUSSION

**Whi5 CDK mutant synthetic lethality with Swi6 CDK mutant**

Whi5 and Swi6 are both targets of CDK activity that results in their inactivation. Whi5 is phosphorylated at Start by Cln:Cdc28 to cause dissociation of Whi5 from SBF and to induce Whi5 export to the cytoplasm (Costanzo et al., 2004; de Bruin et al., 2004). Swi6 is phosphorylated in late G1 by Clb6:Cdc28 on Ser 160, which inhibits the nuclear localization signal of Swi6, resulting in cytoplasmic accumulation of Swi6 (Sidorova et al., 1995). Swi6 has also been implicated as a target of CDK phosphorylation at Start, but the function of those phosphorylations is not understood (Wijnen et al., 2002). The Whi5-12Ala CDK mutant or the Swi6-SA4 CDK mutant have no dramatic phenotype when either is singly expressed in cells. However, the two alleles interact genetically and inhibit cell growth when expressed in cells together.

The synthetic lethality of the Whi5 and Swi6 CDK mutants indicates that their phosphorylation had redundant function. When Swi6-SA4 is present in cells with wild type Whi5, cell growth is not impaired. Likewise, overexpression of Whi5-12Ala CDK mutant in cells with Swi6-WT does not cause a dramatic phenotype. Dramatic inhibition of cell growth is observed only when Whi5-12Ala is overexpressed in Swi6-SA4 cells. Given that Swi6-SA4 cells overexpressing Whi5-12Ala arrest in early G1 as large, unbudded cells, the redundant phosphorylation of Whi5 and Swi6 must facilitate Whi5 inactivation.
**Mutational analysis of Whi5 CDK sites with Swi6-SA4**

We exploited the phenotype of cell lethality caused by overexpression of Whi5-12Ala in Swi6-SA4 cells in a mutational analysis of Whi5 CDK sites to determine which CDK sites are necessary for inactivation. Two models of inactivation were tested here. First, is a specific CDK site or several sites required for Whi5 inactivation? Or second, are a threshold number of CDK sites required for inactivation, without preference for any specific sites? Using this assay, we were able to identify four C-terminal CDK sites of Whi5, sites 8, 9, 10, and 12, that are necessary and sufficient to prevent lethality when Whi5 is overexpressed in combination with Swi6-SA4. Importantly, Whi5 retaining four or more N-terminal CDK sites intact was not sufficient to prevent lethality. This demonstrates that specific CDK sites of Whi5 are required for inactivation, rather than simply a threshold number. Consequently, we can exclude a model similar to that adopted for proteins such as Sic1 that require a threshold number of CDK phosphorylations to induce inactivation without a requirement for specific sites of phosphorylation (Nash et al., 2001). These four phosphorylated residues of Whi5 may function to induce a conformational change or otherwise influence the interaction between Whi5 and SBF components. We conclude that when Whi5 is overexpressed, phosphorylation of Whi5 on four specific CDK sites is required when Swi6 CDK sites are mutated.

Further analysis of the requirement for CDK phosphorylation of Whi5 in the Swi6-SA4 background showed that 3 of the 4 CDK sites are sufficient to
allow cell growth. Analysis in groups of 2 or as single sites demonstrated that no single site or pair of sites was sufficient for viability. We did note that regulation of Whi5 inactivation by phosphorylation is more complex, and that in some combinations N-terminal CDK sites are important. We also note that C-terminal CDK site 11 was not well studied in this assay, and could potentially contribute to Whi5 inactivation in concert with the other C-terminal CDK sites.

Whi5 non-CDK sites do not impact synergism with Swi6-SA4

As a final attempt to assign some function to Whi5 non-CDK sites, the Whi5 non-CDK mutants were also assayed for synthetic lethality with Swi6-SA4. No effect was seen with the Whi5 non-CDK mutants, demonstrating that they are not essential for cell viability when Swi6 CDK sites are mutated. The Whi5-6Ala non-CDK mutant was able to support viability in combination with Swi6-SA4, like Whi5-WT. The Whi5-18Ala CDK and non-CDK mutant had the same phenotype as Whi5-12Ala CDK mutant, showing that the non-CDK sites do not contribute to cell growth in Swi6-SA4 cells.

Functional analysis of the Swi6-SA4 allele

In order to understand the basis for the synthetic lethal interaction between Swi6-SA4 and Whi5-12Ala, we sought to further characterize the Swi6-SA4 mutant. Like wild type Swi6, Swi6-SA4 was capable of rescuing the large cell size of swi6Δ cells. The Swi6 CDK mutant is able to function like
wild type with respect to its regulation of cell size. In analysis of SBF and MBF transcript levels after mating pheromone arrest and release, we found that induction of $CLN1$, $SVS1$, and $RNR1$ mRNA transcripts was the same for Swi6-WT and Swi6-SA4. Thus, Swi6-SA4 does not appear to induce aberrant transcription or disrupt the balance between SBF and MBF transcription. When expressed in otherwise wild type cells, Swi6-SA4 functions similar to wild type Swi6.

**Analysis of Whi5 overexpression**

Because cell cycle regulation in Swi6-SA4 cells appeared to be normal, we turned our attention to analysis of overexpressed Whi5-12Ala to attempt to gain insight into the basis for their cumulative lethality. While expression of Whi5-12Ala at physiologic levels does not inhibit cell cycle progression and is able to rescue the $whi5\Delta$ small cell size, overexpression of Whi5-12Ala, like overexpression of Whi5-WT, causes a G1 delay. Because overexpression of either Whi5-WT or Whi5-12Ala caused a G1 delay in wild type cells, we tested the phenotype of overexpression in Swi6-SA4 cells. Again, overexpression of either Whi5-WT or Whi5-12Ala in Swi6-SA4 cells caused an accumulation of cells in G1. The Whi5-WT Swi6-SA4 cells were able to escape the arrest and resume dividing within 24 hours, but the Whi5-12Ala Swi6-SA4 cells arrested in G1 and died. The Whi5-WT Swi6-SA4 cells did not exit the arrest in a synchronized fashion, however, making comparisons between Whi5-WT and Whi5-12Ala in Swi6 SA4 cells in cell cycle progression analyses difficult.
In an attempt to better study the differences between Swi6-SA4 cells expressing either Whi5-WT or Whi5-12Ala, we synchronized cells with mating pheromone and assayed budding upon release. Wild type cells and Swi6-SA4 cells budded in a timely manner, while cells overexpressing Whi5-WT or Whi5-12Ala were delayed in budding. Cells expressing Swi6-SA4 with either overexpressed Whi5-WT or Whi5-12Ala did not bud to any significant extent in the time period monitored. This indicates that overexpression of Whi5 in the Swi6-SA4 background inhibits release after cell synchronization in G1. This is true for both Whi5-WT and Whi5-12Ala, however, leaving no distinguishing difference between the two alleles. Despite the dramatic difference in cell growth with the assay of lethality on solid media, we cannot examine differences between Swi6-SA4 cells overexpressing Whi5-WT or Whi5-12Ala using a standard cell synchronization experiment.

Further analysis of Whi5 CDK mutants and Swi6-SA4

Two additional approaches will be presented in an attempt to understand the relationship between Swi6-SA4 and Whi5-12Ala. First, localization of Whi5 CDK mutants identified in the mutational analysis of Whi5 interaction with Swi6-SA4 will be examined. Because Swi6-SA4 is localized to the nucleus, nuclear retention of Whi5-12Ala or other CDK mutants identified may be the cause of the synthetic lethal interaction. Additionally, expression at physiologic levels of Whi5 will be examined in combination with Swi6-SA4.
Without the effects of overexpression, analysis of cell cycle phenotypes of Swi6-SA4 and Whi5-12Ala are facilitated.

Chapters 2-6, in part, have been submitted for publication in the journal Eukaryotic Cell. Michelle Wagner, Marcus Smolka, Rob de Bruin, Huilin Zhou, Curt Wittenberg, and Steven Dowdy. The dissertation author was the primary author of this paper.
CHAPTER 5

Localization of Whi5 Phosphorylation Mutants
INTRODUCTION

Phosphorylation of Whi5 on four C-terminal CDK phosphorylation sites is essential for cell viability when Whi5 is overexpressed in the background of the Swi6-SA4 CDK site mutation. Analysis of Swi6-SA4 alone indicates it is able to function normally in transcriptional induction and cell size regulation. Analysis of overexpression of Whi5-WT and Whi5 CDK mutants in otherwise wild type cells causes a G1 cell cycle delay. The effect of the G1 delay caused by Whi5 overexpression may be insurmountable by Swi6-SA4 cells. However, this does not explain why Swi6-SA4 cells are viable with Whi5-WT but dramatically cease cell division with Whi5-12Ala. We were not able to discern any differences between Swi6-SA4 cells overexpressing Whi5-WT versus Whi5-12Ala, except that Swi6-SA4 cells with Whi5-WT eventually escaped the G1 delay induced by Whi5 overexpression while Whi5-12Ala cells remained arrested.

We next examine cellular localization of Whi5 and Whi5 CDK mutants to determine if mis-localization of the Whi5 CDK mutants is contributing to lethality observed in Swi6-SA4 cells. Costanzo et al. showed that wild type Whi5 is shuttled between the nucleus and the cytoplasm at distinct stages of the cell cycle (Costanzo et al., 2004). In early G1, Whi5 is nuclear, consistent with its role as a direct transcriptional repressor. At Start, Whi5 is exported to the cytoplasm, and this nuclear export requires CDK phosphorylation (Costanzo et al., 2004). It is thought that at the end of mitosis Whi5 is
dephosphorylated and allowed to return to the nucleus. Indeed, in cells with a cdc28-4 temperature sensitive allele, Whi5 shuttled normally at the permissive temperature, but rapidly returned to the nucleus at the restrictive temperature (Costanzo et al., 2004). This demonstrates the importance of CDK phosphorylation in regulation of Whi5 nuclear export.

In this section, we assess localization of Whi5-WT, 12Ala, and CDK mutants of the four critical sites required for cell viability in combination with Swi6-SA4. Cellular localization of the Whi5 proteins was determined in both wild type cells and Swi6-SA4 cells. The results indicate that Swi6-SA4 does not impact localization of Whi5, although we demonstrate that MSN5 and SWI6 are required genes for Whi5 nuclear export. Our analysis also indicates that the localization of Whi5-CDK mutants is not the critical factor that impacts viability of Swi6-SA4 cells.
RESULTS

Localization of Whi5-WT and Whi5-12Ala CDK mutant

In wild type cells, Whi5 localization is nuclear in early G1, then exported to the cytoplasm just prior to budding (Costanzo et al., 2004). Since Swi6-SA4 is constitutively localized in the nucleus (Sidorova et al., 1995), we hypothesized that Whi5 CDK mutants that caused lethality with Swi6-SA4 might also have defects in nuclear export. Thus, localization of GFP tagged Whi5 was examined. As previously reported (Costanzo et al., 2004), Whi5-WT-GFP was nuclear in unbudded cells and cells just exiting mitosis, but was relocated to the cytoplasm in budded cells (Fig. 5.1, A). However, Whi5-12Ala-GFP was localized to the nucleus in both unbudded and budded cells (Fig. 5.1, B), confirming that CDK phosphorylation is required for Whi5 nuclear export (Costanzo et al., 2004).

Localization of Whi5-WT-GFP and Whi5-12Ala-GFP was also examined in Swi6-SA4 cells. The localization was identical to that observed in wild type cells. Wild type or Swi6-SA4 cells expressing Whi5-WT-GFP had Whi5 nuclear localization in 98-100% of unbudded cells. In budded cells with wild type or Swi6-SA4 background, Whi5-WT-GFP was cytoplasmic in 100% of cells. Analysis of localization of Whi5-12Ala-GFP revealed it was 100% nuclear localized in both unbudded and budded cells of wild type and Swi6-SA4 background. Quantification of the percent of budded cells with cytoplasmic Whi5 is shown in Fig. 5.2, C.
FIG. 5.1 Localization of Whi5-GFP wild type and 12Ala CDK mutant. (A) Representative photos of Whi5-WT-GFP in unbudded and budded cells. (B) Representative photos of Whi5-12Ala-GFP in unbudded and budded cells. DIC and fluorescent images are overlaid.
FIG. 5.2. Localization of Whi5 4Ala and 8Ala\(^1\) phosphorylation mutants. 
(A) Representative photos of Whi5-4Ala-GFP and (B) Whi5-8Ala\(^1\)-GFP in 
budded and unbudded cells. (C) Graph showing quantification of 
cytoplasmic localization of Whi5-WT and CDK mutants in budded cells.
Localization of Whi5-4Ala and Whi5-8Ala\(^1\) CDK mutants

We next examined localization of GFP tagged Whi5 CDK mutants 4Ala and 8Ala\(^1\). Overexpression of Whi5-4Ala, containing mutations of CDK sites 8, 9, 10, and 12, caused lethality in Swi6-SA4 cells, while overexpression of Whi5-8Ala\(^1\) with CDK sites 8, 9, 10, and 12 wild type, allowed for viability with Swi6-SA4 (Fig. 4.3). If localization of Whi5 was affecting viability of Swi6-SA4 cells, then these Whi5 CDK mutants should affect localization. In wild type or Swi6-SA4 cells, the localization of Whi5-4Ala-GFP was nuclear in 98-100% of unbudded cells (Fig. 5.2, A), similar to Whi5-WT. In budded cells of wild type or Swi6-SA4 background, Whi5-4Ala was cytoplasmic in only 15-16% (Fig. 5.2, A, 2\(^{nd}\) panel and Fig. 5.2, C). Whi5-4Ala remained nuclear in 84-85% of budded cells, in contrast to Whi5-WT. This indicates that CDK sites 8, 9, 10 and 12 are not absolutely necessary for nuclear export of Whi5, but do have some role in promoting export because cytoplasmic localization in budded cells was much lower than with Whi5-WT.

The other CDK mutant examined, Whi5-8Ala\(^1\)-GFP, was nuclear in 94-98% of unbudded cells in either a wild type or Swi6-SA4 background (Fig. 5.2, B), similar to Whi5-WT. Whi5-8Ala\(^1\)-GFP was cytoplasmic in 35-36% of budded cells in wild type and Swi6-SA4 cells (Fig. 5.2 B and C). Whi5-8Ala\(^1\)-GFP remained nuclear in 64-65% of budded cells, both wild type and Swi6-SA4. These observations suggest that in wild type or SWI6-SA4 cells, Whi5 C-terminal CDK sites 8, 9, 10, and 12 are not entirely sufficient for nuclear export, but are able to promote cytoplasmic localization in a portion of budded
cells. While CDK sites 8, 9, 10, and 12 have a role in Whi5 nuclear export, they are neither necessary nor sufficient, indicating that other CDK sites of Whi5 are involved in promoting nuclear export and cytoplasmic localization after cells pass Start.

**MSN5 and SWI6 are required for Whi5 nuclear export**

Swi6 nuclear export is dependent on the karyopherin Msn5 (Queralt and Igual, 2003). Because Swi6 and Whi5 interact (Costanzo et al., 2004; de Bruin et al., 2004), we examined whether Whi5 nuclear export was also Msn5 dependent. In *msn5Δ* cells, GFP tagged Whi5-WT was constitutively nuclear, even in budded cells (Fig. 5.3, A). This indicates that Msn5 is required for Whi5 nuclear export. Whi5-WT-GFP was also nuclear in *swi6Δ* cells (Fig. 5.3, B), suggesting that Swi6 is also required for Whi5 nuclear export. We attempted to determine if Msn5 physically interacts with Whi5 by performing co-immunoprecipitation experiments. We could not detect interaction with Msn5 and Whi5, although we were able to confirm interaction between Msn5 and Swi6 (data not shown). This indicates that Msn5 may be influencing Whi5 nuclear export through an indirect mechanism.

Both Whi5 CDK phosphorylation and Swi6 CDK phosphorylation are required for their respective nuclear export. Msn5 has also been shown to export several other phosphorylated proteins (Boustany and Cyert, 2002; DeVit and Johnston, 1999; Kaffman et al., 1998; Queralt and Igual, 2003), indicating phosphorylation may be a prerequisite for Msn5 binding and export.
FIG. 5.3. Localization of Whi5 is MSN5 and SWI6 dependent.
(A) Representative photos of msn5Δ cells expressing Whi5-GFP from the GAL1 promoter. (B) Representative photos of swi6Δ cells expressing Whi5-GFP from the GAL1 promoter. (C) Western blot of immunopurified and phosphatase treated Whi5-WT-13xMyc from wild type cells, msn5Δ, and swi6Δ cells. Arrows indicate the different phosphorylated forms of Whi5.
We examined phosphorylation status of Whi5-WT immunoprecipitated from \textit{msn5}Δ and \textit{swi6}Δ cells compared to wild type cells. In all three samples, Whi5 was both hypo- and hyper-phosphorylated, and sensitive to phosphatase treatment (Fig. 5.3, C). Whi5 was phosphorylated in both \textit{msn5}Δ and \textit{swi6}Δ cells, indicating that the lack of Whi5 export in those cells is not due to lack of CDK phosphorylation.

**Overexpression of MSN5 does not induce ectopic export of Whi5**

If Msn5 is directly responsible for Whi5 nuclear export, we hypothesized that Msn5 overexpression would be able to induce Whi5-WT nuclear export prematurely, and could also result in Whi5-12Ala nuclear export. Msn5 was placed under control of the \textit{GAL1} promoter at the \textit{MSN5} endogenous locus (Longtine et al., 1998). Growth of cells in galactose to induce Msn5 overexpression was not sufficient to induce ectopic nuclear export of Whi5-WT-GFP, as its localization remained nuclear in unbudded cells (Fig. 5.4, A, 1\textsuperscript{st} panel). Whi5 nuclear export occurred normally, as localization remained cytoplasmic in budded cells overexpressing Msn5 (Fig. 5.4, A, 2\textsuperscript{nd} panel). Overexpression of Msn5 was unable to induce export of Whi5-12Ala-GFP, as its localization remained nuclear in both unbudded and budded cells (Fig. 5.4, A, 3\textsuperscript{rd} and 4\textsuperscript{th} panels). In these experiments, Whi5-GFP expression was also driven from the \textit{GAL1} promoter, potentially obscuring any effect of extra Msn5. However, wild type levels of Msn5 are able to support complete nuclear export of \textit{GAL1} produced Whi5-WT-GFP (Fig. 5.1, A), indicating overexpressed Whi5
FIG. 5.4. GAL1-MSN5 does not delocalize Whi5 or rescue Swi6-SA4 Whi5-12Ala cells.
(A) Localization of Whi5-GFP expressed from the GAL1 promoter in cells also expressing Msn5 from the GAL1 promoter. Representative photos of Whi5-WT-GFP (panels 1 and 2) and Whi5-12Ala-GFP (panels 3 and 4) are shown.
(B) Expression of GAL-MSN5 in swi6Δ cells with Swi6-SA4 and either empty vector, Whi5-WT, or Whi5-12Ala expressed from the GAL1 promoter.
is not overwhelming the normal export machinery; thus, any hypermorphic function of GAL1-MSN5 would have been detected.

Although GAL1-MSN5 was not able to induce ectopic export of Whi5-WT or 12Ala, we assayed its ability to affect viability of Whi5-12Ala Swi6-SA4 CDK mutant cells. Congruent with its inability to force nuclear export, GAL1-MSN5 was unable to rescue the growth defect of GAL1-WHI5-12A SWI6-SA4 co-expressing cells (Fig. 5.4, B). Even with overexpressed Msn5, Whi5-12Ala Swi6-SA4 cells are unable to grow. As a whole, these results indicate that Msn5 is necessary for Whi5 nuclear export, but perhaps not via a direct mechanism.
DISCUSSION

Localization of Whi5 CDK mutants

CDK phosphorylation of Swi6 participates in the inactivation of Swi6 and SBF transcription by inducing cytoplasmic localization (Geymonat et al., 2004; Sidorova et al., 1995). Swi6 Serine-160 is adjacent to a nuclear localization sequence and inhibits nuclear import once phosphorylated. When Ser-160 is mutated to alanine, as it is in the Swi6-SA4 mutant, this results in constitutive nuclear localization of Swi6 (Sidorova et al., 1995). Whi5 is also exported from the nucleus upon CDK phosphorylation, and a C-terminal phosphorylation mutant of Whi5 (CDK sites 7-12 alanine) is largely nuclear (Costanzo et al., 2004). Our analysis of Whi5-GFP localization concurs with previously published results, in that wild type Whi5 is nuclear in unbudded cells, and relocates to the cytoplasm in budded cells. The Whi5-12Ala CDK mutant was constitutively localized to the nucleus, confirming the observation that CDK phosphorylation is required for nuclear export.

Because both Whi5-12Ala and Swi6-SA4 are both nuclear proteins, we hypothesized that constitutive nuclear localization of both proteins was contributing to the inhibition of cell growth observed in Swi6-SA4 cells overexpressing Whi5-12Ala. We assessed the localization of two other Whi5 CDK mutants, Whi5-4Ala, that caused cell lethality in combination with Swi6-SA4, and Whi5-8Ala', that did not inhibit cell growth with Swi6-SA4. Analysis of Whi5-GFP localization shows the four C-terminal sites (8, 9, 10, and 12) are
involved in nuclear export, as Whi5-4A was unable to be exported to the cytoplasm in 85% of budded cells. However, the four sites are not sufficient to control cellular localization, as Whi5-8Ala\(^1\) (sites 8, 9, 10, and 12 wild type) remained nuclear in two thirds of budded cells. Additionally, cellular localization of Whi5-WT and Whi5 CDK mutants was the same for wild type cells or Swi6-SA4 cells. This suggests that the four CDK sites implicated in Whi5 inactivation in Swi6-SA4 cells differ from the CDK sites required to induce Whi5 nuclear export. This also implies that constitutive nuclear localization is not the chief cause of cell lethality in Swi6-SA4 cells with Whi5 CDK mutants. Because the Whi5 mutant with CDK sites 7-12 mutated to alanine remains largely nuclear (Costanzo et al., 2004; Skotheim et al., 2008), these data now suggest a function for CDK sites 7 and 11 in Whi5 export. These data also indicate that Whi5 dissociation from SBF and its subsequent export to the cytoplasm are distinct steps requiring phosphorylation of different CDK sites.

**Requirement for Msn5 and Swi6 in Whi5 nuclear export**

In the course of examining localization of Whi5-CDK mutants, we also determined that Msn5 and Swi6 are essential for Whi5 nuclear export. Msn5 is a karyopherin that is involved in the nuclear export of several proteins, including Swi6 (Strom and Weis, 2001). Msn5 is also known to export phosphorylated proteins (Boustany and Cyert, 2002; DeVit and Johnston, 1999; Kaffman et al., 1998; Queralt and Igual, 2003). Whi5-WT-GFP is
constitutively localized to the nucleus in \(msn5\Delta\) as well as in \(swi6\Delta\) cells, indicating that both proteins have a role in Whi5 nuclear export. Whi5 remains phosphorylated in \(msn5\Delta\) cells and in \(swi6\Delta\) cells, indicating that lack of phosphorylation is not the cause of the nuclear retention of Whi5 in these cells.

In order to determine if Msn5 interacts directly with Whi5 to induce its nuclear export, we performed co-immunopurification experiments and analyzed the effect of Msn5 overexpression on Whi5 localization. While we were able to detect Msn5 interaction with Swi6, we could not co-immunoprecipitate Msn5 and Whi5. This does not preclude the possibility that the two interact however. When Msn5 was overexpressed from the \(GAL1\) promoter, it was not able to promote aberrant localization of Whi5. In \(GAL1-MSN5\) cells, similar to wild type cells, Whi5-WT-GFP was nuclear in unbudded cells and Whi5-12Ala-GFP was nuclear in all cells. Additionally, overexpression of Msn5 did not change the cell lethality phenotype of Swi6-SA4 Whi5-12Ala cells. This suggests that Msn5, even at super-physiologic levels, will not support Whi5 nuclear export if Whi5 is not phosphorylated.

The requirement for Swi6 in Whi5 nuclear export suggests a model wherein the two proteins are exported together. Whi5 and Swi6 have been shown to bind throughout the cell cycle (de Bruin et al., 2004), meaning their interaction is not limited to binding at SBF promoters in early G1. While Whi5 is released from promoters at Start and exported to the cytoplasm, Swi6 remains bound to promoters to promote transcription until late G1/S phase.
before it is phosphorylated and accumulates in the cytoplasm (Queralt and Igual, 2003; Sidorova et al., 1995). If Whi5 was exported with Swi6, it would require some exchange at promoters, where either inactivated Whi5 bound another free Swi6 protein, or both Whi5 and Swi6 release from promoters together and another Swi6 molecule binds the SBF promoter. These models remain to be tested. We also note that Whi5 was exported to the cytoplasm normally in Swi6-SA4 cells. We hypothesize that Swi6-SA4 could still perform its role in assisting in Whi5 nuclear export because its CDK mutations inhibit a nuclear localization sequence. It is likely that Swi6-SA4 is exported to the cytoplasm, with Whi5, but is then immediately targeted for nuclear localization, explaining its appearance in the nucleus of budded cells. This model also needs to be tested. Finally, we note that Whi5 binding to Swi6 is not sufficient to support nuclear export. Whi5-12Ala readily bound Swi6 in co-immunoprecipitation studies, but remains localized to the nucleus. This indicates that phosphorylation of Whi5 is required for nuclear export in addition to binding Swi6.

**Inhibition of cell growth by overexpressed Whi5-12Ala and Swi6-SA4**

These experiments show that nuclear localization of Whi5 CDK mutants is not the determining factor in causing cell lethality with Swi6-SA4. Because we were not able to perform cell cycle analysis experiments with overexpressed Whi5, we refocused our efforts on analysis of Whi5-12Ala and
Swi6-SA4 when Whi5 is expressed at physiologic levels. The results of the experiments with physiologic expression will be presented in the next chapter.

Chapters 2-6, in part, have been submitted for publication in the journal Eukaryotic Cell. Michelle Wagner, Marcus Smolka, Rob de Bruin, Huilin Zhou, Curt Wittenberg, and Steven Dowdy. The dissertation author was the primary author of this paper.
CHAPTER 6

Whi5 and Swi6 Phosphorylation in Cell Size Regulation
INTRODUCTION

To determine the basis of the lethality caused by overexpression of Whi5-12Ala in Swi6-SA4 cells we have examined the function of Swi6-SA4 alone, the effect of overexpression of Whi5 alone, and the localization of Whi5 CDK mutants in wild type and Swi6-SA4 cells. In analysis of Whi5 localization, Whi5-12Ala is constitutively localized to the nucleus. Being that Swi6-SA4 is also continually imported into the nucleus, we tested the hypothesis that Whi5-12Ala Swi6-SA4 cells were unable to divide due to interference in cell cycle progression caused by continued nuclear localization of the two proteins. We showed that the CDK mutant Whi5-4Ala, that causes a growth defect in Swi6-SA4 cells like Whi5-12Ala, is largely nuclear in budded cells. However, the converse mutant, Whi5-8Ala¹, with the four critical CDK sites wild type that allows for normal growth with Swi6-SA4, was still largely localized to the nucleus. While the localization of both the 4Ala and 8Ala¹ mutants is also partially cytoplasmic, the cellular localization of the mutants does not correspond with their ability to inhibit or foster growth in Swi6-SA4 cells.

Because we did not gain significant understanding of the cell growth inhibition caused by overexpression of Whi5 CDK mutant in Swi6 CDK mutant cells by analysis of Swi6-SA4, Whi5 overexpression, or Whi5 CDK mutant localization, we have concentrated our efforts on analysis of Whi5 at physiologic levels. We would like to determine whether the four CDK sites identified in the mutational analysis of Whi5 bear any significance in a normal
cell cycle. Expression from the GAL1 promoter is useful in that it can be easily induced or repressed by different carbon sources. However, when induced, the GAL1 promoter is extremely productive, resulting in protein levels that can be several times higher than levels generated from an endogenous promoter. To study Whi5 CDK mutant expression at physiologic levels we utilized a fragment of the WHI5 promoter cloned into a yeast CEN plasmid. Expression from this promoter produces similar levels of protein as the endogenous WHI5 locus and is several times lower than expression induced by GAL1 (See Fig. 3.2).

Here we show that in combination with Swi6-SA4, expression of Whi5-12Ala at physiologic levels is not lethal. In fact, cells expressing both mutants are viable and grow at rates similar to cells expressing both wild type versions of Swi6 and Whi5. We do report, however, that the Swi6-SA4 cells expressing Whi5-12Ala at physiologic levels have a defect in regulation of cell size. This cell size defect is also seen with the Whi5-4Ala mutant, indicating the four CDK sites important for cell viability when Whi5 is overexpressed with Swi6-SA4 are also important for regulation of cell cycle when Whi5 is expressed at physiologic levels.
RESULTS

Growth rates of Whi5 CDK mutant Swi6-SA4 mutant cells

Four C-terminal CDK phosphorylation sites of Whi5 were necessary and sufficient for cell viability when Whi5 was overexpressed with Swi6-SA4 (Fig. 4.3). Overexpression of Whi5-WT or Whi5-12Ala in Swi6-SA4 cells both resulted in a G1 accumulation of cells (Fig. 4.6), making analysis of the nature of cell lethality caused by Whi5-12Ala or Whi5-4Ala (sites 8, 9, 10, 12 Ala) in Swi6-SA4 cells difficult to investigate. To determine how the four C-terminal CDK sites of Whi5 affect cell cycle progression, WHI5-WT and CDK site mutant alleles were expressed at physiologic levels from the WHI5 promoter on CEN plasmids in combination with SWI6-SA4. The WHI5 promoter used was the 545 bp fragment 5' of the WHI5 open reading frame. In contrast to cells overexpressing Whi5-12Ala from the GAL1 promoter (as in Fig. 4.3), cells expressing Whi5-12Ala and Swi6-SA4 from their own promoters at physiologic levels were viable and grew at rates similar to cells expressing Whi5-WT and Swi6-WT (Fig. 6.1) and had normal cell cycle profiles (data not shown). As controls, cells containing only one of the phosphorylation mutants, Swi6-SA4 or Whi5-12Ala, also grew at similar rates over the course of several hours and had normal cell cycle profiles (Fig. 6.1). This indicates that at physiologic expression levels, phosphorylation of Whi5 or Swi6 is not required for cell viability or normal cell cycle progression. The redundant requirement for Whi5
FIG. 6.1. Growth Rates of Whi5 CDK mutant Swi6-SA4 mutant cells. Growth rates were examined for whi5Δswi6Δ double knockout cells with CEN plasmids containing Whi5-WT or 12Ala under control of the 545bp WHI5 promoter and 2µ plasmids with Swi6-WT or Swi6-SA4 under control of the SWI6 promoter. Overnight cultures of cells were diluted into fresh media then OD600 readings were taken at given times.
or Swi6 phosphorylation is only essential for cell viability when Whi5 is overexpressed.

**Whi5 CDK mutant Swi6-SA4 mutant cells release from mating pheromone arrest**

We have demonstrated that cells expressing Whi5-12Ala and Swi6-SA4 at physiologic levels are able to grow normally in asynchronous culture. We also examined their ability to enter the cell cycle after a mating pheromone induced G1 arrest. When cells were synchronized in G1 with mating pheromone and released, *WHI5-12A SWI6-SA4* cells budded at the same time and rate as *WHI5-WT SWI6-WT* cells (Fig. 6.2). Control cells with single mutants Whi5-12Ala or Swi6-SA4 also budded at the same rate after release. All cultures began budding between 20 and 30 minutes, and reached peak amounts of budding around 70 minutes (Fig. 6.2). Phosphorylation of Whi5 and/or Swi6 is not necessary for cell cycle progression after mating factor induced G1 arrest and subsequent release. The timing of budding of Whi5-12Ala Swi6-SA4 double mutant cells was the same as cells with both wild type alleles, indicating that SBF transcription and other cell cycle regulatory events are occurring normally in the double mutant cells.

**Whi5 CDK mutant affects cell size with Swi6-SA4**

Thus far we have demonstrated that cells expressing Whi5-12Ala and Swi6-SA4 at physiologic levels divide and cycle normally, in contrast to results
FIG. 6.2. Whi5 Swi6 double mutant cells release from mating factor arrest.
Cell synchronization with alpha factor was performed for whi5Δswi6Δ double knockout cells with CEN plasmids containing Whi5-WT or 12Ala under control of the 545bp WHI5 promoter and 2µ plasmids with Swi6-WT or Swi6-SA4 under control of the SWI6 promoter. Log phase cultures were treated with alpha factor, washed, released into fresh media and budding index examined at given time intervals.
observed when Whi5 is overexpressed in the Swi6-SA4 background. We next
examined the cell size distribution of the double mutant cells. Although the
cells displayed no overt growth defects, Whi5-12Ala SWI6-SA4 co-expressing
cells were large in size (Fig. 6.3). Wild type cells, shown in red, have a normal
cell size distribution in comparison to smaller whi5Δ cells, shown in green, or
larger swi6Δ cells, shown in blue. Cells with both wild type Whi5 and Swi6,
shown in black, have a profile consistent with wild type cells. Cells with single
mutants Whi5-12Ala or Swi6-SA4, shown in pink and light blue, respectively,
also have a size distribution of cells with both wild type alleles. This indicates
that only in combination do the physiologic levels of Whi5-12Ala and Swi6-SA4
cause a larger cell size.

**Whi5-4Ala CDK mutant causes a large cell size with Swi6-SA4**

Because Whi5-12Ala at physiologic levels in combination with Swi6-
SA4 resulted in a larger cell size, we measured the cell size distribution of
Whi5-4Ala and Whi5-8Ala CDK mutants in Swi6-SA4 cells. As controls,
samples were compared to wild type cells (shown as a red tracing), whi5Δ
cells with a small cell size (shown as a green tracing), and swi6Δ cells with a
larger cell size (shown as a brown tracing) (Fig. 6.4). In this experiment, the
mean cell volume (fL) of cells co-expressing Whi5-12Ala and Swi6-SA4
(shown as a light blue tracing) was 67.6 fL, 40% larger than cells expressing
wild type Whi5 and Swi6 (shown as a dark blue tracing) with a mean of 48.2
fL, again demonstrating that phosphorylation of either Swi6 or Whi5 is
FIG. 6.3. Whi5-12Ala Swi6-SA4 double mutant cells have a large cell size.

Cell size distribution was analyzed for whi5Δswi6Δ cells with CEN plasmids containing Whi5-WT or 12Ala under control of the 545bp WHI5 promoter and 2μ plasmids with Swi6-WT or Swi6-SA4 under control of the SWI6 promoter. These are compared to wild type cells (BY4741) and single mutant whi5Δ or swi6Δ cells as controls.
FIG. 6.4. Whi5-4A CDK mutant causes a large cell size with Swi6-SA4. Cell size analysis was performed for \(\text{whi}5\Delta\text{swi}6\Delta\) cells with CEN plasmids containing Whi5-WT or 12Ala under control of the 545bp \(\text{WHI5}\) promoter and 2\(\mu\) plasmids with Swi6-WT or Swi6-SA4 under control of the \(\text{SWI6}\) promoter. Cells with Swi6-SA4 and Whi5-4Ala and Whi5-8Ala\(^1\) were also examined.
necessary for regulation of cell size (Fig. 6.4). Cells with \textit{WHI5-WT} and \textit{SWI6-SA4} (shown as a pink tracing) or cells with \textit{WHI5-12Ala SWI6-WT} (shown as a black tracing) were similar in size distribution to \textit{WHI5-WT SWI6-WT} cells (shown as a dark blue tracing), confirming that the effect on cell size is not observed in cells with either mutant alone.

We then analyzed the cell size distribution of Whi5 CDK mutants. When expressed with Swi6-WT, Whi5-4Ala and Whi5-8Ala\(^1\) cells were similar in size to Whi5-WT and Whi5-12Ala (data not shown). In contrast, cells expressing Swi6-SA4 with Whi5-4Ala (CDK sites 8, 9, 10, 12 Ala) at physiologic levels were larger in size (shown as a yellow tracing) with a mean size of 62.0 fl, similar to Swi6-SA4 Whi5-12Ala cells (Fig. 4.6). Conversely, the size distribution of Swi6-SA4 cells with Whi5-8Ala\(^1\) (cdk sites 8, 9, 10, 12 WT) (shown as a purple tracing) with a mean size of 54.6, was similar to that of cells containing both wild type alleles. These results demonstrate that in combination with Swi6-SA4, phosphorylation of Whi5 C-terminal CDK sites (8, 9, 10, and 12) is necessary and sufficient for maintenance of cell size. This result highlights the importance of the four C-terminal CDK sites identified as essential for viability when Whi5 is overexpressed with Swi6-SA4 (Fig. 4.3), and demonstrates that they remain essential for regulation when Whi5 is expressed at physiologic levels in combination with Swi6-SA4.
DISCUSSION

**Whi5 CDK mutant at physiologic levels in Swi6-SA4 cells**

Despite the dramatic cell cycle phenotype of *Swi6-SA4* cells when Whi5-12Ala is overexpressed, CDK phosphorylation of Whi5 and Swi6 is not essential for viability when Whi5 is expressed at physiologic levels. Cells expressing both Swi6-SA4 and Whi5-12Ala under control of their own promoters are completely viable, grow at normal rates, and enter the cell cycle after mating pheromone induced G1 arrest with normal kinetics. These observations contrast dramatically with cells overexpressing Whi5 or Whi5-12Ala, which exhibit a G1 accumulation of cells and delay of cell cycle entry after pheromone release. The only apparent defect of physiologic expression of Whi5-12Ala with Swi6-SA4 is a large cell size, suggesting that cells are delayed in passing Start and spend more time in early G1 when Whi5 and Swi6 cannot be phosphorylated. This is in contrast to cells containing either phosphorylation mutant alone; cells expressing Swi6-SA4 alone or Whi5-12Ala at physiologic levels are normal in size.

**Four Whi5 C-terminal CDK sites (8, 9, 10, and 12) are necessary for cell size regulation with Swi6-SA4**

Because there was a biological difference with respect to cell size distribution observed between Swi6-SA4 cells expressing either Whi5-WT or Whi5-12Ala, we exploited the opportunity to test other Whi5 CDK mutants for
the ability to cause an atypical cell size in combination with Swi6-SA4. Our results indicate that the four Whi5 CDK sites (8, 9, 10, and 12) identified in the overexpression assay with Swi6-SA4 are also necessary and sufficient to promote cell size regulation in combination with Swi6-SA4. We showed that \textit{WHI5-4Ala SWI6-SA4} cells were equivalent in size to \textit{WHI5-12Ala SWI6-SA4} cells with a large cell size phenotype. Conversely, \textit{WHI5-8Ala} \(^\dagger\) (CDK sites 8, 9, 10, and 12 wild type) cells were similar in size to \textit{WHI5-WT} when combined with \textit{SWI6-SA4}. The effect of these mutants on cell size indicates that the four specific CDK sites are necessary and sufficient for the timing of Whi5 inactivation and SBF activation, even at physiologic expression levels. In the future, we propose to demonstrate that the large cell size observed in the double mutant cells is due to a delay in SBF transcriptional activation.

**Implications of redundant phosphorylation in regulation of cell size**

These data also demonstrate that Whi5 and Swi6 are not essential targets of Cln-Cdc28 for cell viability when expressed at physiologic levels. The redundant phosphorylation of Swi6 or the four sites of Whi5 is only required for proper coordination of cell size with cell cycle entry, likely impacting timing of transcriptional activation. In the absence of available CDK sites on Whi5 or Swi6, cells must use a mechanism other than CDK phosphorylation to activate SBF transcription. It is possible that the cell cycle activator Bck2 plays a role under these conditions, given its ability to activate SBF transcription independently of Cdc28 activity (Di Como et al., 1995;
Wijnen and Futcher, 1999). Or alternatively, because MBF transcription was normal in cells expressing Swi6-SA4, it is likely that MBF transcription still occurs normally in Swi6-SA4 Whi5-12Ala cells. This normal regulation of MBF could support cell cycle progression given that many G1 target genes are regulated by both SBF and MBF. The mechanism of Whi5 inactivation and stimulation of SBF transcription in the absence of Whi5 or Swi6 CDK phosphorylation remains to be determined.

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CHAPTER 7

Regulation of G1 Cell Cycle Progression by

Reactive Oxygen Species
INTRODUCTION

Cell cycle progression in yeast

Cell division is a carefully regulated process where cells assess extracellular signals and intracellular checkpoints before making the decision to grow and divide. In yeast, environmental nutrient availability regulates accumulation of cellular mass to control progression through early G1 (Hartwell et al., 1974; Rupes, 2002). Nutrient deprivation or a failure to reach a critical cell size results in an early G1 arrest (Barbet et al., 1996; Rupes, 2002; Werner-Washburne et al., 1993). Thus, cell cycle progression through G1 is dependent on cell growth and nutrient availability. However, it is unknown if cells gauge cell size by measuring their volume, mass, translation rate and/or metabolic capacity, or how the signal of sufficient size is relayed to promote cell cycle progression (Jorgensen and Tyers, 2004).

Cell cycle progression from G1 to S phase is regulated by the activities of the Cln:Cdc28 cyclin:cyclin dependant kinase (CDK) complexes (Mendenhall and Hodge, 1998). In early G1 phase, Cln3:Cdc28 complexes become active when nutrients are sufficient (Polymenis and Schmidt, 1997). At the early to late G1 transition, termed Start, Cln3:Cdc28 phosphorylates and inactivates Whi5, an inhibitor of the SBF (Swi6/Swi4) transcriptional complex (Costanzo et al., 2004; de Bruin et al., 2004). Once active, the SBF and MBF (Swi6/Mbp1) complexes initiate expression of a suite of genes, including the late G1 cyclins, Cln1 and Cln2, as well as the S phase cyclins.
Cln1 and Cln2 bind Cdc28 and phosphorylate substrates to regulate progression through late G1 (Hadwiger et al., 1989) and initiation of budding. Clb5 and Clb6 cyclins also associate with Cdc28 in late G1, but are bound and inhibited by Sic1 (Schwob et al., 1994). The Cln1/2:Cdc28 complexes phosphorylate and inactivate Sic1p, targeting it for degradation (Nash et al., 2001). This activates Clb5:Cdc28 and Clb6:Cdc28 to initiate DNA replication. Although the molecular machinery of cell cycle progression through G1 is understood, the initial signals that relay parameters of cell size and metabolic capacity remain unknown.

**Reactive oxygen species as signaling molecules**

High levels of oxygen free radicals are known to induce oxidative stress and DNA damage in cells (Huang and Kolodner, 2005; Salmon et al., 2004). However, low levels of endogenously generated reactive oxygen species (ROS) have been implicated in diverse signaling pathways (Finkel, 2003; Lambeth, 2004). For example, nitric oxide is a well studied endogenously produced ROS that stimulates vascular vasodilation (Ignarro, 2002). Likewise, in mammalian cells, growth factor stimulation induces a burst of ROS coincident with entry into the cell cycle (Burdon, 1995). The sources of reactive oxygen generated within a cell include mitochondrial electron transport chain leakage (Boveris, 1976; Boveris and Chance, 1973), peroxisome lipid metabolism (Hiltunen et al., 2003), and in mammalian cells,
receptor activated NADPH oxidases (Lambeth, 2004). In both mammalian cells and in yeast, the generation of ROS in the cell is balanced out with antioxidant enzymes and compounds. Superoxide dismutases (SOD1 and SOD2) reduce superoxide anion to hydrogen peroxide, while catalases (CTT1 and CTA1) and peroxidases reduce hydrogen peroxide to water (Jamieson, 1998; Sauer et al., 2001b). Additional molecules such as glutathione, thioredoxin (TRX1, TRX2), and glutaredoxin (GRX1, GRX2) serve to efficiently scavenge reactive oxygen (Grant, 2001). The ability of ROS to be rapidly generated and easily degraded makes them ideal candidates for signaling molecules.

**Links between ROS and cell cycle progression**

It was recently reported that endogenous ROS are required for progression through late G1 in mammalian cells, specifically to inactivate APC<sup>Cdh1</sup> before S phase entry (Havens et al., 2006). It has also been shown that mitochondrial generated ROS are required for G1 to S phase progression in *Drosophila* (Owusu-Ansah et al., 2008). To understand the relationship between endogenous reactive oxygen species and cell cycle progression in the yeast *S. cerevisiae*, cells were treated with the antioxidant compound Tempol to reduce ROS levels. Tempol is a membrane-permeable piperidine nitroxide that mimics SOD activity by catalytically reducing superoxide (Krishna and Samuni, 1994; Samuni et al., 1990). Compounds having the same reactive group, Tempo and Tempamine, were also used. Tempol
treatment of cells resulted in decreased cell proliferation due to the arrest of cells in early G1 phase, with low CDK activity and before induction of SBF and MBF transcription. Although reduction of ROS results in an early G1 arrest in yeast and a late G1 arrest in mammalian cells, this work demonstrates that, similar to mammalian cells and Drosophila, endogenous ROS are necessary for G1 to S phase cell cycle progression in yeast.
RESULTS

Endogenous levels of ROS are reduced by antioxidant treatment

To understand the relationship between endogenous reactive oxygen species and cell cycle progression in yeast, cells were treated with the antioxidant compound Tempol to reduce ROS levels (Krishna and Samuni, 1994; Samuni et al., 1990). Incubation of an asynchronous culture of yeast with Tempol resulted in a decrease in the level of endogenous ROS to 46% of levels observed in a normal log phase culture (Fig. 7.1). In comparison, treatment with 0.5% hydrogen peroxide, levels known to cause DNA damage, resulted in a 92% increase in intracellular ROS. This demonstrates that, similar to results in mammalian cells (Havens et al., 2006), unperturbed asynchronously dividing yeast cells maintain a detectable level of endogenous ROS below levels that cause cell damage. Additionally, application of the antioxidant reduces endogenous cellular ROS levels by half.

ROS are required for G1 cell cycle progression

We analyzed cells treated with the antioxidant Tempol to assess the effect of reducing endogenous ROS on cell cycle progression. Reduction of cellular ROS levels by Tempol resulted in a dramatic decrease in cell proliferation (Fig. 7.2, A). Cultures treated with Tempol did not significantly increase in OD 600, a measure of culture turbidity correlating with cell number (Fig. 7.2, A), or actual cell number over the course of 7 hours. In the same
FIG. 7.1. Antioxidant treatment lowers ROS levels
Relative ROS levels as measured by fluorescence of dihydroethidium in live yeast cells either untreated, Tempol treated, or hydrogen peroxide treated. Fluorescent levels were normalized to values of the untreated sample.
FIG. 7.2. Antioxidant treatment causes a growth defect and G1 arrest
(A) Cell growth of untreated cells compared to Tempol treated cells. (B) Cell cycle profiles of untreated, asynchronous cells, and Tempol cells arrested in G1. Cells were fixed for cell cycle analysis after 5 hours of growth.
time period, an untreated asynchronous sample of cells divided at a normal rate, increasing OD600 as well as cell number (Fig. 7.2, A).

Because proliferation of an asynchronous culture was inhibited upon antioxidant treatment, we analyzed the cell cycle profiles of Tempol treated cultures to determine if cells were arrested during a particular cell cycle stage. Analysis of cell cycle profiles of DNA content with flow cytometry showed that Tempol treated cells had a higher percentage of cells in G1 phase (90%) compared to the untreated asynchronous sample (40%) (Fig. 7.2, B). This demonstrated that reduction of endogenous ROS by Tempol impairs cell proliferation by arresting cells in G1 phase. Tempol added to an asynchronous culture caused a dramatic G1 arrest. Therefore, cells in S phase, G2, or mitosis completed their current cell cycle before arresting in the next G1. Thus, endogenously generated ROS are required specifically in G1 phase and do not effect progression through other phases of the cell cycle.

To ensure the G1 cell cycle arrest phenotype caused by Tempol was due to its catalytic ability to reduce ROS, and not via a nonspecific mechanism, other catalytic antioxidant compounds were also tested for their ability to inhibit cell cycle progression. Tempo and Tempamine are two compounds similar to Tempol, all having the same reactive nitrooxide group on a six member ring (see Fig. 7.3 for compound structures). Similar to Tempol treatment, addition Tempo or Tempamine to asynchronous cultures inhibited the culture from increasing in OD 600. This indicates the compounds were also causing an inhibition of cell division (Fig. 7.4, A). The Tempo and
FIG. 7.3. Chemical structures of antioxidant compounds. (A) Tempol, also known as 4-hydroxy-tempo. (B) Tempamine, also known as 4-amino-tempo. (C) Tempo. All three compounds contain the reactive nitroxide group.
FIG. 7.4. Other antioxidants cause a growth defect and G1 arrest.
(A) Cell growth of untreated cells compared to cells treated with Tempol, Tempo, or Tempamine. (B) Cell cycle profiles of untreated asynchronous cells, and Tempol, Tempo, or Tempamine treated cells arrested in G1.
Tempamine treated cultures were analyzed by flow cytometry for DNA content to determine if they arrested at a specific cell cycle stage. Similar to Tempol treatment of asynchronous cells, Tempo or Tempamine treated cells accumulated in the G1 phase of the cell cycle (Fig. 7.4, B). This demonstrates that two additional compounds with catalytic ability to reduce ROS caused a growth inhibition where cells accumulate in G1 phase. This also indicates that Tempol, Tempamine, and Tempo act via their common nitroxide reactive group. These data confirm that endogenous levels of ROS are required for G1 phase cell cycle progression.

**Antioxidant arrested cells are large in size and unbudded**

To characterize the G1 arrest induced by Tempol treatment, we next analyzed cell size and budding index. In an asynchronous untreated culture, cells that have completed S phase were approximately twice as large as the mean size of cells in G1 phase (Fig. 7.5, A). Cells arrested in G1 with Tempol treatment were larger than G1 cells in the asynchronous population, and were similar in size to the untreated G2/M population (Fig. 7.5, A). This indicates that while cell cycle progression was inhibited in G1 by Tempol treatment, cellular growth continued. This demonstrated that the reduction of ROS by treatment of asynchronous cultures with Tempol is inhibiting cell cycle progression in G1 downstream of pathways promoting cell growth.

Yeast cells begin to form a bud that will become a daughter cell after cells pass through G1. The presence or absence of a bud, and the size of the
FIG. 7.5. Antioxidant treated cells are large in size and unbudded. (A) Relative cell size of untreated G1 and G2/M cells compared to Tempol treated cells. (B) Representative photos of untreated, asynchronous cells and Tempol cells arrested in G1.
bud, can indicate the cell cycle stage of an individual cell. The majority of cells arrested by Tempol treatment were unbudded (90% unbudded), compared to 5% unbudded cells in control cultures (Fig. 7.5, B). This indicates the Tempol treated cells have not progressed beyond G1 phase to bud and corroborates the DNA profiles showing a G1 arrest.

**Antioxidant treated cells arrest in early G1 before Start**

In further characterization of the antioxidant induced G1 arrest, we next addressed the question of whether Tempol treatment arrests cells in early G1, or in late G1 after Start. As normal cells progress past Start into late G1, a transcriptional program controlled by SBF and MBF transcription factor complexes is induced. Examination of the levels of SBF and MBF target gene transcripts will indicate if they are repressed, as in early G1 cells, or if they are induced, as in late G1 cells. Whole genome microarray analysis was performed comparing the transcript levels in Tempol arrested G1 cells to transcript levels in an asynchronously dividing population. While levels of housekeeping genes were at similar levels between the two samples, levels of SBF and MBF target genes were decreased in Tempol treated cells (Fig. 7.6). A representative list of 24 known SBF and MBF targets demonstrated the mRNA transcripts were repressed between 2 and 10 fold in Tempol cells as compared to untreated cells (Fig. 7.6). Tempol treatment of cells thus results in a G1 arrest before induction of SBF and MBF transcription at Start.
Fig. 7.6. Ordered List of SBF and MBF transcript levels in treated cells. Transcripts in yellow are housekeeping genes, those in blue are SBF and MBF targets down regulated in Tempol treated cells.
While analysis of cell cycle regulated transcripts indicated that Tempol treated cells arrest before Start, we also analyzed the levels of cell cycle regulated proteins to confirm the early G1 arrest. The SBF targets and G1 cyclins Cln1 and Cln2 were absent from arrested cells, and no associated Cdc28 kinase activity was detected (Fig. 7.7, A). Additionally, the S phase cyclin Clb5 was at low levels and had little associated kinase activity. However, Cdc28 kinase was present at normal levels (Fig. 7.7, A). The absence of G1 cyclins and lack of SBF and MBF induced transcription indicate Tempol treatment arrests yeast in early G1 before Start.

**Analysis of Sic1 CDK inhibitor in Tempol treated cells**

Because we observed low Clb5 protein levels and no associated kinase activity in Tempol arrested cells, we also examined levels of the CDK inhibitor Sic1. The Sic1 protein is normally present throughout early and late G1 to bind and inhibit Clb5/6-Cdc28, until Sic1 is phosphorylated by Cln1/2:Cdc28 in late G1 and targeted for degradation (Nash et al., 2001; Schwob et al., 1994). As determined by immunoblot analysis, the level of Sic1 protein in Tempol arrested cells was at lower levels than in untreated cells (Fig. 7.7, B). However, Cln1 and Cln2 are absent from Tempol arrested cells; consequently, they cannot target Sic1 for degradation. The cyclin Pcl1 in complex with Pho85 is also capable of phosphorylation of Sic1, targeting it for degradation (Nishizawa et al., 1998). Analysis of Pcl1 levels indicated, like Cln1 and Cln2, it was absent from Tempol treated cells (Fig. 7.7, B). Because *PCL1* is also
FIG. 7.7. Antioxidant treatment causes an early G1 arrest.
(A) Immunoblot analysis and kinase assays of G1 cyclins Cln1, Cln2, & Clb5 from untreated and Tempol treated cells. (B) Immunoblot analysis of Sic1 and Pcl1 protein levels in untreated and Tempol treated cells. (C) Reverse transcriptase PCR of SIC1 transcript levels in untreated and Tempol treated cells. Reactions were done with and without reverse transcriptase (RT).
an SBF target gene, this is not unexpected. Due to the absence of Cln1, Cln2, and Pcl1 cyclins, Sic1 must be targeted for degradation by some unknown mechanism in the Tempol arrested cells. Alternatively, *SIC1* may fail to be transcribed or translated in the arrested cells.

We analyzed the levels of *SIC1* transcript to determine if lack of transcription was the cause of low levels of Sic1 protein in Tempol treated cells. *SIC1* is normally transcribed in late mitosis and early G1, under control of the Swi5 transcription factor. Whole genome microarray data showed a slight decrease in *SIC1* transcript levels; in Tempol treated cells *SIC1* was at 70% of levels in untreated cells. We performed reverse transcriptase PCR on untreated and Tempol treated cells, that verified *SIC1* transcript was slightly decreased in Tempol arrested cells compared to untreated cells (Fig. 7.7, C). RT-PCR for the *ACT1* transcript was utilized as a control.

Because we observed SIC1 transcripts at slightly lower levels after Tempol treatment, we also analyzed transcript levels of other Swi5 target genes to determine if Swi5 dependent transcription was repressed in Tempol arrested cells (Knapp et al., 1996). We utilized the microarray data to examine transcript levels of *PCL9*, *EGT2*, and *HO* that are all Swi5 target genes. In addition to lower *SIC1* mRNA levels, *PCL9* levels were at 50%, *EGT2* levels were at 77%, and *HO* transcript levels were at 89% after Tempol treatment compared to the transcript levels from untreated levels. This indicated that other Swi5 transcriptional targets in addition to *SIC1* are slightly decreased. It is unknown whether the slight decrease observed in Swi5 transcription is
solely responsible for the lack of Sic1 protein. It remains to be determined if
SIC1 mRNA is untranslated or if Sic1 protein is targeted for degradation in
Tempol treated cells.

Ectopic overexpression of G1 cyclins does not rescue Tempol induced
cell cycle arrest

The observations above demonstrated that Tempol treatment arrests
cells in early G1 with low levels of cyclin proteins and undetectable
Cln1/Cln2/Clb5-Cdc28 associated kinase activity. We hypothesized
antioxidant treated cells may be arrested before Start due to absence of robust
cyclin:Cdc28 kinase activity. We sought to override the Tempol induced G1
arrest by overexpression of G1 cyclins. To determine if forced expression of
G1 cyclins was able to promote progression through G1 in Tempol treated
cells, cyclins Cln1, Cln2, Clb5, or the stable Cln2^{4T3S} under the control of the
GAL1 promoter were used. In various experiments, cyclin expression was
induced with galactose either before Tempol treatment, simultaneous with
Tempol treatment (on solid media), or after the Tempol treatment. Cell cycle
DNA profiles (data not shown) and absence of growth on plates (Fig. 7.8)
showed cyclin overexpression could not overcome the G1 arrest induced by
Tempol treatment. Cells overexpressing Cln1, Cln2, Cln2-1, or Clb5 still arrest
in G1 and are not able to form colonies in the presence of Tempol (Fig. 7.8).
This indicates that the absence of G1 cyclins is not the only factor keeping
Tempol treated cells arrested in G1.
FIG. 7.8. Tempol arrest is not rescued by overexpression of G1 cyclins. Cells containing CLN1, CLN2, the stabilized CLN2-1, and CLB5 under control of the GAL1 promoter were plated on glucose or galactose in the presence or absence of the antioxidant Tempol.
Genetic selection for Tempol resistant mutants

To identify the genetic pathways involved in ROS regulation of G1 cell cycle progression, a genetic selection was performed to identify mutants that were resistant to Tempol induced G1 cell cycle arrest. The haploid Mat-a *Saccharomyces* Genome Deletion collection was plated in the presence of the antioxidant Tempol. The wild type parent strain (*BY4741*) failed to grow on Tempol plates. In contrast, 18 mutant strains were identified that grow in the presence of Tempol (Fig. 7.9). Four of the 18 strains were weakly resistant to Tempol, while the other 12 grew robustly on Tempol plates. The classes of genes represented by the resistant mutants included those involved in protein degradation, DNA damage repair, regulation of transcription and translation, and cell wall maintenance, among others.

Further analysis was undertaken to verify that the specific gene deletion in each mutant strain was responsible for the phenotype of Tempol resistance. We observed that the gene deletion did not always segregate with Tempol resistance in tetrad analysis. Additionally, reintroduction of the wild type genes into the deletion strains failed to confer Tempol sensitivity. Therefore we concluded that the specific gene deletions were not responsible for the resistance phenotype and secondary mutations of other genes must be involved. Unfortunately, attempts to identify the causative genes of the Tempol resistance phenotype by complementation with a plasmid library of genomic DNA were unsuccessful. We believe that the Tempol resistant phenotype of these mutants is due to multiple genetic lesions within each
FIG. 7.9. Deletion mutants resistant to cell cycle inhibition by Tempol.
strain. Additionally, because antioxidants cause a cell cycle arrest, the functions of essential genes or pathways are most likely being inhibited. The requirement for essential genes in mediating the regulation of cell cycle progression by ROS has not been assayed. Future experiments are needed to better understand how Tempol is inhibiting cell cycle progression and what genes confer the requirement for ROS in cell cycle progression.
DISCUSSION

Reactive oxygen species are required for G1 cell cycle progression

In this study we demonstrate that endogenous levels of reactive oxygen species are required for cell cycle progression in yeast. Treatment of cells with the antioxidant compound Tempol resulted in lower endogenous ROS levels caused a cell cycle arrest cells in G1 phase. Two other related antioxidant compounds, Tempo and Tempamine, also caused a G1 cell cycle inhibition. Like mammalian cells and Drosophila (Havens et al., 2006; Owusu-Ansah et al., 2008), native levels of ROS are essential for transition through G1 phase. Also similar to mammalian cells, yeast cells arrest in G1 but continue to grow in size when treated with the antioxidant compound Tempol. Nutrient signaling to induce cell growth and accumulation of mass is an early event in G1 cell cycle regulation that is not inhibited by antioxidants, and thus does not require ROS.

ROS are required to pass Start

Analysis of cells arrested in G1 by Tempol showed they arrest in early G1 before Start. Cells arrest unbudded, an indication they arrest before S phase initiation. Microarray analysis of Tempol treated cells showed SBF and MBF regulated transcription at Start was greatly reduced compared to untreated cells. Additionally, levels of G1 cyclins and associated kinase activity were low in arrested cells. We conclude that Tempol treated cells are
arrested in early G1 phase, downstream of pathways promoting increase in cell size. This differs from the results reported for mammalian cells, where ROS are specifically required at the late G1 to S phase transition to inactivate APC\textsuperscript{Cdhl} and allow accumulation of Cyclin A and other proteins essential for S phase. Despite the differences between the arrest in yeast and mammalian cells, the basic requirement for ROS in regulation of cell cycle progression is conserved.

**Overriding the requirement for ROS in G1 cell cycle progression**

To attempt to understand how reactive oxygen species affect cell cycle progression, we used two different methods to attempt to overcome the Tempol imposed G1 arrest. First, we overexpressed G1 cyclins, hypothesizing the G1 arrest was due to lack of G1 associated kinase activity. The induction of cyclin gene expression did not result in resistance to the G1 arrest induced by Tempol. This indicates there are other factors or pathways involved in activation of cell cycle progression that are inhibited by Tempol, and therefore require endogenous levels of ROS to function.

In a second attempt to override the cell cycle inhibitory effects of Tempol, we screened the haploid non-essential gene deletion library for strains that were resistant to the Tempol arrest. This selection identified 18 resistant strains. Although the gene deletions present in the strains were not responsible for the Tempol resistance phenotype, these mutants show that the requirement for ROS in cell cycle progression is not absolute and can be
bypassed in some strains. The genetic basis for the ability of the identified strains to circumvent the requirement for ROS in G1 is unknown and remains to be determined.

**Function of ROS in cell cycle progression**

High levels of reactive oxygen species are associated with DNA damage and cellular stress. We show in this study that lower levels of ROS exist endogenously in cells and are in fact essential for cell division. We have shown that ROS are required for transition past Start, but have not identified the critical target of ROS or the reason why it is essential in cell cycle progression. Recent studies have shown ribosome content is crucial for cells to sense size and signal cell cycle progression when cells reach the critical cell size (Jorgensen et al., 2004). Reduction of ROS by Tempol could be interfering with this signaling, as the Tempol treated cells are large in size, indicating the critical cell size has been achieved, but do not pass Start and initiate cell division. Likewise, endogenous generation of ROS, perhaps as metabolic byproducts, could be a signal the cell uses to sense size and robust metabolism and trigger Start. The requirement for ROS in G1 cell cycle progression is conserved between mammalian cells and yeast, but the source of the ROS and the mechanism of ROS signaling to cell cycle regulators remains to be determined in both yeast and mammalian cells.

Chapter 7, in part will be submitted for publication in the journal Cell Cycle. Michelle Wagner and Steven Dowdy. The dissertation author was the primary author of this paper.
CHAPTER 8

Discussion and Conclusions
DISCUSSION AND CONCLUSIONS

Whi5 CDK and non-CDK phosphorylation

Whi5 is a negative regulator of G1 cell cycle progression that is inactivated by CDK phosphorylation at Start (Costanzo et al., 2004; de Bruin et al., 2004). Whi5 contains 12 putative CDK phosphorylation sites. However, it has remained unclear if specific CDK phosphorylation sites on Whi5 are utilized for inactivation or if a critical phosphorylation threshold of randomly distributed CDK sites induces inactivation. In this study of Whi5 regulation by phosphorylation, we determined that Whi5 is always found phosphorylated in vivo, but phosphorylation of Whi5 is not necessary for function unless Swi6 phosphorylation is also impaired. We identified four specific CDK sites of Whi5 that are essential for Whi5 inactivation and regulation of cell size in combination with the Swi6 CDK phosphorylation mutant.

In our initial analysis of Whi5 phosphorylation, we found that Whi5 is phosphorylated in vivo on 10 CDK sites and 6 novel, non-CDK sites. We also observed that Whi5 is found hypo-phosphorylated at all stages of the cell cycle, including early G1. We did not observe un-phosphorylated Whi5, even in mating pheromone arrested cells. Analysis of Whi5 phosphorylation mutants and in vivo orthophosphate labeling of Whi5 indicated that besides the 12 putative CDK sites and the 6 non-CDK sites, there are no other significant phosphorylated residues on Whi5. With a clear-cut analysis of
Whi5 phosphorylation events, we next investigated the function of phosphorylation.

Activation of CDK activity correlates with inactivation of Whi5 and its dissociation from promoters *in vivo* and *in vitro* (Costanzo et al., 2004; de Bruin et al., 2004). This suggested that phosphorylation of Whi5 CDK sites is important in SBF activation and cell cycle progression. To test this, we analyzed the function of the Whi5-12Ala CDK mutant. Whi5-12Ala, expressed from the *WHI5*, *MET3*, or *GAL1* promoters, was able to rescue the small cell size of *whi5*Δ cells. The normal cell size of Whi5-12Ala cells indicates that CDK phosphorylation of Whi5 is not required for cell cycle progression. This suggests that CDK activity is targeting other factors, likely Swi6 or possibly Swi4, to promote SBF activation in the absence of Whi5 CDK phosphorylation sites. Our cell size analysis results differ from those of de Bruin et al, who report that Whi5-7Ala expressed from the *MET3* promoter results in a delay in cell cycle progression, with a synchronized population of elutriated cells inducing maximum SBF transcription at ~10% larger cell size (de Bruin et al., 2004). The differences in results may be due to use of different strain backgrounds, the use of the heterologous promoter, or differences in experimental design. Yet, the effect of Whi5-7Ala was not as dramatic as would be predicted if Whi5 phosphorylation was required for SBF activation.

While the cell size analysis demonstrated that CDK sites of Whi5 are not required for Whi5 inactivation, we also tested whether phosphorylation is required for Whi5 activation. Because Whi5 was found hypo-phosphorylated
in early G1, and Rb is hypo-phosphorylated in early G1 when bound to E2F complexes, we hypothesized that hypo-phosphorylation of Whi5 could be a prerequisite for binding and inhibiting SBF. We analyzed both CDK and non-CDK mutants of Whi5 for their ability to cause lethality in \textit{cln3}\Delta cells when overexpressed as a measure of Whi5 inhibitory activity. Similar to wild type Whi5, the CDK mutant 12Ala, non-CDK mutant 6Ala, and combination CDK and non-CDK mutant 18Ala were all found to cause lethality in \textit{cln3}\Delta cells when overexpressed. This indicates phosphorylation of Whi5 is not required for its inhibitory function. As overexpression of proteins can potentially lead to a gain of function, we also assayed the function of Whi5 phosphorylation mutants when expressed at more physiologic levels from the \textit{MET3} promoter in \textit{cln3}\Delta bck2\Delta whi5\Delta triple knockout cells. Induction of physiologic expression of Whi5 wild type caused cell lethality, as \textit{cln3}\Delta bck2\Delta cells are inviable with a functional Whi5. Similar to wild type Whi5, all phosphorylation mutants tested caused lethality when expressed at physiologic levels in \textit{cln3}\Delta bck2\Delta whi5\Delta triple knockout cells. Despite lacking functional phosphorylation sites, Whi5 was able to effectively inhibit cell cycle progression and induce lethality in the absence of two essential activators of Start. Therefore, phosphorylation of Whi5 CDK or non-CDK sites is not essential for Whi5 inhibitory function (see Fig. 8.1 for model).

We continued the analysis of Whi5 non-CDK sites in an attempt to determine their function. We determined that they are not required for cell size
FIG. 8.1. Models of Whi5 and Swi6 phosphorylation at Start.

Repressed
Whi5 phosphorylation is not required to inhibit SBF

Activated
Whi5 phosphorylated on 4 sites when Swi6 cannot be phosphorylated

Activated
Swi6 phosphorylated when Whi5 cannot be phosphorylated
regulation, activation of Whi5, or inactivation of Whi5. Whi5 non-CDK sites remained phosphorylated in the Whi5-12Ala CDK mutant, and were also phosphorylated in early G1 cells lacking Cln-Cdc28 kinase activity. Thus, non-CDK phosphorylation is independent of CDK activity. Additionally, the non-CDK Whi5 phosphorylation mutant remained phosphorylated on CDK sites. Therefore, non-CDK phosphorylation is not a prerequisite for subsequent targeting of Whi5 by CDKs. However, we were unable to attribute any function to the non-CDK phosphorylation sites of Whi5. Five out of 6 of the non-CDK sites are followed by acidic residues and conform to the consensus sequences for casein kinase I or casein kinase II (CKA1/2) (Ptacek et al., 2005), which is known to target transcription factors and RNA polymerases (Glover, 1998). Perhaps Whi5 is phosphorylated on non-CDK sites as a result of binding SBF transcription factor complexes. Further experiments are needed to explore the biological function of Whi5 non-CDK phosphorylation and identify the kinase(s) responsible.

**Redundancy of Whi5 and Swi6 Phosphorylation**

In contrast to the finding that Whi5 phosphorylation is not essential for cell size regulation at physiologic expression levels, phosphorylation of Whi5 and Swi6 becomes essential when Whi5 is overexpressed, as phosphomutants of these proteins synergize to cause lethality. Swi6 has 5 putative CDK phosphorylation sites; four of the five are in the N-terminal
region, while the fifth CDK site is a part of the ankyrin repeat domain of Swi6. The SWI6-SA4 mutant created by Sidorova, et al., that contains mutations of the four N-terminal CDK sites, does not affect periodic transcription of SBF targets (Sidorova et al., 1995). We confirmed that the Swi6-SA4 mutant was functional, able to compensate for the large cell size of swi6Δ cells, and regulated transcription of SBF and MBF target genes normally after mating pheromone arrest and release.

While Swi6-SA4 alone does not have a significant cell cycle phenotype, Swi6-SA4 cells expressing GAL1-WHI5-12Ala are inviable and arrest as large, elongated, unbudded cells. We exploited this phenotype in a mutational analysis of Whi5 CDK sites and identified four C-terminal CDK sites of Whi5, sites 8, 9, 10, and 12, that are necessary and sufficient to prevent lethality when Whi5 is overexpressed in combination with Swi6-SA4. As a control, Whi5 retaining four or more N-terminal CDK sites intact was not sufficient to prevent lethality. We also determined that of the four critical CDK sites identified, groups of three of the four sites are sufficient for Whi5 inactivation. While the four sites remain important, there is some plasticity in the requirement for all four sites to be functional.

The mutational analysis of Whi5 CDK sites demonstrated four specific CDK sites of Whi5 are required for inactivation, rather than simply a randomly distributed threshold amount of phosphorylation. Accordingly, we exclude a model similar to that adopted for proteins such as Sic1 that require a threshold number of CDK phosphorylations to induce inactivation without a requirement
for specific sites of phosphorylation (Nash et al., 2001). The function of these four phosphorylated residues of Whi5 may serve to inactivate Whi5 by inducing a conformational change or influencing the interaction between Whi5 and SBF components. We conclude that phosphorylation of four specific CDK sites of Whi5 and CDK sites of Swi6 is redundant, and the redundant phosphorylation facilitates Whi5 inactivation and SBF activation (Fig. 8.1).

**Whi5 nuclear localization**

In addition to dissociation from SBF, Whi5 is also exported from the nucleus upon CDK phosphorylation. Consequently, a C-terminal phosphorylation mutant of Whi5 (sites 7-12 alanine) is largely nuclear (Costanzo et al., 2004), but was recently shown to shuttle slowly out of the nucleus (Skotheim et al., 2008), indicating CDK phosphorylation is required for efficient Whi5 nuclear export. In late G1, CDK phosphorylation of Swi6 participates in the inactivation SBF transcription by inducing cytoplasmic localization of Swi6 (Geymonat et al., 2004; Sidorova et al., 1995). When Swi6 CDK sites are mutated to alanine, as in the Swi6-SA4 mutant, this results in continued nuclear localization of Swi6 (Sidorova et al., 1995).

We hypothesized that the lethality observed with Swi6-SA4 cells overexpressing Whi5 CDK phosphorylation mutants might be due to the lack of efficient nuclear export of the two proteins. Therefore, we analyzed the nuclear localization of Whi5 CDK mutants in wild type cells and in Swi6-SA4 cells. Analysis of Whi5-GFP localization shows four C-terminal sites (8, 9, 10,
and 12) are involved in nuclear export, as Whi5-4A was unable to be completely exported to the cytoplasm in budded cells, but are not sufficient to control localization, as Whi5-8Ala\(^1\) (sites 8, 9, 10, and 12 wild type) remained nuclear in two thirds of budded cells. This suggests that the four CDK sites implicated in Whi5 inactivation differ from the CDK sites required to induce robust Whi5 nuclear export. These observations also leave open the possibility that Whi5 nuclear export does not require phosphorylation of specific sites and may be determined by a threshold amount of phosphorylation. This data also indicates that activation of SBF by Whi5 inactivation and Whi5 export to the cytoplasm are distinct steps requiring phosphorylation of different Whi5 CDK sites.

**Physiologic expression of Whi5 CDK mutants with Swi6-SA4**

Because we determined the lethality observed in Swi6-SA4 cells overexpressing Whi5-12Ala was not due to a defect in Swi6-SA4 function or aberrant localization of Whi5, we turned to examination of Whi5 expression at physiologic levels to further investigate the redundant phosphorylation of Whi5 and Swi6. Although *SWI6*-SA4 cells overexpressing Whi5-12Ala dramatically arrest in G1 and are inviable, CDK phosphorylation of Whi5 and Swi6 is not essential for viability when Whi5 is expressed at physiologic levels. Swi6-SA4 cells expressing Whi5-12Ala under control of the *WHI5* promoter are viable, divide at normal rates, and enter the cell cycle after mating pheromone induced G1 arrest with wild type kinetics. Their only apparent defect is a large
cell size, suggesting that cells remain in G1 longer and are delayed in passing Start when Whi5 and Swi6 cannot be phosphorylated.

In analysis at physiologic levels, the four Whi5 CDK sites (8, 9, 10, and 12) identified in the overexpression assay with Swi6-SA4 are also necessary and sufficient to promote cell size regulation, as \( WHI5\text{-}4\text{Ala} \) \( SWI6\text{-}SA4 \) cells were equivalent in size to \( WHI5\text{-}12\text{Ala} \) \( SWI6\text{-}SA4 \) cells. Conversely, \( WHI5\text{-}8\text{Ala} \) (CDK sites 8, 9, 10, and 12 wild type) cells were similar in size to \( WHI5\text{-}WT \) when combined with \( SWI6\text{-}SA4 \). The effect of these mutants on cell size indicates that the four specific CDK sites are important for the timing of Whi5 inactivation, even at physiologic expression levels. However, these data also imply that Whi5 and Swi6 are not essential targets of Cln-Cdc28 for SBF activation when expressed at physiologic levels. Because phosphorylation of Swi6 or the four sites of Whi5 is only required for proper coordination of cell size with cell cycle entry, the redundant phosphorylation acts as a mechanism to activate cell cycle progression at Start once cells have reached a critical cell size. Although proper timing of Start is abolished in the absence of available CDK sites on Whi5 or Swi6, cells must utilize a failsafe mechanism other than CDK phosphorylation to activate SBF transcription. It is possible that the cell cycle activator Bck2 plays a role under these conditions, given its ability to activate SBF transcription independently of Cdc28 activity (Di Como et al., 1995; Wijnen and Futcher, 1999).

**Parallels between Whi5 and Rb**
The pathway of transcriptional activation in yeast is functionally similar to the mechanism of cell cycle entry in mammalian cells. Whi5 is a functional analog of the retinoblastoma tumor suppressor protein (Rb) that also represses transcription in early G1. Previous studies from our laboratory show that Rb bound to E2F transcription factors in early G1 is hypo-phosphorylated on CDK phosphorylation sites (Ezhovsky et al., 1997). Whi5 is similarly hypo-phosphorylated in early G1, but we found that hypo-phosphorylation is not necessary for its repressive function. Further experiments are needed to determine the requirement for Rb hypo-phosphorylation in repression of E2F promoters. Additionally, further investigation is needed to determine what function Whi5 hypo-phosphorylation may serve.

In our analysis of Whi5 phosphorylation, we also addressed whether Whi5 inactivation required specific phosphorylations or responded to a threshold amount of phosphorylation. We demonstrated that four specific CDK sites were necessary and sufficient for Whi5 inactivation and impacted regulation of cell size. In contrast, analyses of Rb phosphorylation suggest it is not chiefly regulated by specific phosphorylation events. For example, phosphopeptide maps of hyper-phosphorylated Rb during late G1 show a nearly identical pattern to maps of hypo-phosphorylated Rb found in early G1, with the main difference being a 5-10 fold increase in phosphorylation occupancy (Lees et al., 1991; Mittnacht et al., 1994). This indicates that individual CDK sites are targeted equally in both Rb hypo- and hyper-phosphorylated forms. Because hypo-phosphorylated Rb is active and hyper-
phosphorylated Rb inactive, this suggests specific sites do not have a significant function and the amount of phosphorylation determines Rb activity. Taken together, these observations suggest a model of Rb regulation where inactivation by hyper-phosphorylation is achieved by phosphorylation of a threshold number of CDK sites (Ezhevsky et al., 2001). In contrast to that model for Rb, we identified specific CDK sites that function in Whi5 inactivation and regulation of cell size, and show Whi5 is not regulated by a threshold model of phosphorylation. Because the regulatory pathways of cell cycle entry are largely conserved between yeast and mammalian cells, the mechanism of regulation by specific phosphorylation observed for Whi5 and the relative unimportance of Whi5 hypo-phosphorylation suggest further investigation of Rb regulation by phosphorylation in mammalian systems is warranted.

**Regulation of G1 cell cycle progression by Reactive Oxygen Species**

In the course of our investigations into regulation of cell cycle progression by Whi5 phosphorylation, we also examined a relatively novel hypothesis that reactive oxygen species (ROS) play an important role in cell cycle regulation. Our laboratory recently published findings that mammalian cells produce endogenous levels of ROS that oscillate with each cell cycle (Havens et al., 2006). When the levels of endogenously produced ROS are quenched by exogenous addition of antioxidant compounds, cells arrest in G1 phase. In mammalian cells, ectopically lowering ROS levels arrested cells after the Restriction Point in late G1 phase, before inactivation of APC\textsuperscript{Cdh1} and
before accumulation of Cyclin A protein. We investigated whether the requirement for endogenous ROS in G1 phase cell cycle progression was conserved in yeast. We planned to genetically dissect the pathway ROS utilize to regulate G1 in the more genetically tractable single celled yeast to gain insight into the regulation of G1 by ROS in mammalian cells.

Yeast cells, like mammalian cells, maintain an endogenous level of ROS when unperturbed in asynchronous growth. This level of endogenous ROS is much lower than levels of ROS normally associated with DNA damage and cellular stress. Application of the antioxidant Tempol to asynchronous yeast was able to lower the endogenous ROS levels. Tempol treatment also caused a cell division defect, with cells arresting their cell cycle in G1. The G1 arrest phenotype was also replicated with use of similar antioxidant compounds, Tempo and Tempamine. This indicated the observed G1 arrest was due to the antioxidant property of the common reactive nitroxide group present in all three compounds, and not a nonspecific effect of one compound.

The initial results indicate that yeast cells, like mammalian cells, endogenously produce ROS that is required at a certain level for G1 cell cycle progression.

We also characterized the G1 arrest in yeast to determine if antioxidant treatment arrested yeast in late G1 similar to mammalian cells. Analysis of SBF and MBF transcript levels indicated they were low in Tempol treated cells. Additionally, protein levels of SBF and MBF targets Cln1/2 and Clb5 were low. Therefore, the Tempol treatment arrested the yeast in early G1, before induction of SBF and MBF transcription at Start. Analysis of Sic1 and other
Swi5 regulated transcripts indicated they were slightly repressed in Tempol treated cells. These data show antioxidant treatment of yeast results in an early G1 arrest, before Start, in contrast to the late G1 arrest observed in mammalian cells.

Although the yeast arrest at a different point in G1 after antioxidant treatment than do mammalian cells, we continued with a genetic approach to identify mutants resistant to Tempol arrest. We screened the haploid, non-essential *Saccharomyces* Gene Deletion Collection and identified 18 strains able to grow in the presence of Tempol. Unfortunately, the gene deletions were not solely responsible for the ability to continue cell division in the presence of Tempol. This indicates there are secondary mutations in the deletion strains that confer resistance to Tempol. We hypothesize that several genes may be mutated in the Tempol resistance phenotype, because attempts to identify the additional mutant gene in resistant strains by complementation with a plasmid library of genomic DNA were unsuccessful. Nevertheless, the identification of resistant mutants shows that the requirement for ROS in cell cycle progression is not absolute and can be transversed by mutant cells. In conclusion, the requirement for ROS in G1 cell cycle progression is conserved between yeast and mammalian cells although the pathways that regulate ROS integration into cell cycle progression may differ.
Future Directions

ROS and G1 cell cycle progression

There are several questions that remain to be investigated with relation to the requirement for ROS in G1 cell cycle regulation. First, the origin of the ROS and the particular species of reactive oxygen that is required for cell cycle progression is unknown. The sources of reactive oxygen generated within a cell include mitochondrial electron transport chain leakage (Boveris, 1976; Boveris and Chance, 1973), peroxisome lipid metabolism (Hiltunen et al., 2003), and in mammalian cells, receptor activated NADPH oxidases (Lambeth, 2004). The generation of ROS in the cell is balanced out with antioxidant enzymes and endogenous compounds, such as superoxide dismutase, catalase, peroxidase, glutathione, thioredoxin, and glutaredoxin that serve to efficiently scavenge reactive oxygen (Grant, 2001). The ability of ROS to be rapidly generated and easily degraded makes them ideal candidates for signaling molecules. Drugs that inhibit production of ROS within the cell, like DPI or rotenone, that inhibit mitochondrial electron transport, or mutant strains with temperature sensitive alleles of genes involved in endogenous production of ROS could be utilized to examine the critical origin of the ROS. Identification of the source of ROS could give insight into the species of ROS that is relevant to cell cycle progression.
In addition to the origin or source of ROS within the cell, it remains to be determined how ROS regulates cell cycle progression in G1. The molecule or pathway that ROS is targeting is unknown. ROS have been shown to affect the functional activity of phosphatases, kinases, and transcription factors (Filomeni et al., 2005; Sauer et al., 2001a; Sauer et al., 2001b). Additionally, SUMOylation, a covalent modification of proteins that affects protein activity, is inhibited in conditions of low endogenous ROS levels (Bosis and Melchior, 2006). It is possible that ROS is affecting a redox-sensitive protein, pathway, or protein modification as a signal to activate cell cycle progression. In mammalian cells, it is possible that ROS is affecting APC^{Cdh1} and the timing of its inactivation. Because yeast arrest in early G1, ROS may target pathways essential for signaling critical cell size to trigger Start, such as ribosome biosynthesis or translation rate. Those and other pathways could be examined in Tempol arrested cells to determine if they are inhibited upon antioxidant treatment.

Finally, regulation of Sic1 protein levels in Tempol arrested cells warrants further investigation. Tempol arrested cells in early G1 have low Sic1 levels. Sic1 is normally phosphorylated and targeted for degradation in late G1 by Cln1/2-Cdc28, or Pcl1-Pho85 kinase activities (Nishizawa et al., 1998; Schwob et al., 1994). However, those cyclin-CDK complexes are not present in Tempol arrested cells, so the phosphorylation of Sic1 to trigger degradation cannot occur. We hypothesize that other methods of regulation are responsible for the low levels of Sic1. It is known that presence of Sic1 in G1
requires both transcription and translation (Visintin et al., 1998). The transcription of SIC1 is at slightly lower levels in antioxidant treated cells, but it is unknown if that reduction in transcript is responsible for the subsequent absence of Sic1 protein. To determine if lack of translation is responsible for low Sic1 in Tempol arrested cells, the translation of SIC1 mRNA could be investigated, perhaps by examining polysome profiles. Since SIC1 transcript is present at least to some degree, it is possible that Tempol is interfering with protein synthesis on a larger scale. Incorporation of $^{35}$S-methionine could be measured in Tempol cells in comparison to untreated cells to determine if protein translation is inhibited. Additionally, if Sic1 is being translated, proteosome inhibitor drugs or temperature sensitive proteosome subunits could be used to determine if Sic1 protein is being targeted for degradation. In this case, if Sic1 were targeted for degradation in Tempol arrested cells, it would be by a novel mechanism independent of Cln1/2 or Pcl1. Overall, establishment of the concept that yeast cells and mammalian cells both require endogenous levels of ROS for cell cycle progression is a novel finding that raises several related queries.

**Regulation of Whi5 by Phosphorylation**

Investigation into the function of Whi5 and how it is influenced by modification by phosphorylation also raises several questions for future investigations. We discovered 6 novel non-CDK phosphorylation events on Whi5 that are not required for regulation of cell size, Whi5 inhibitory activity, or
for subsequent CDK phosphorylation. Several possible functions exist for the non-CDK sites of Whi5. We preliminarily investigated whether non-CDK phosphorylation is involved in glucose signaling. When cells are shifted from poor media to glucose rich media, they inhibit the cell cycle in G1, specifically inhibiting CLN1 transcription, allowing the cell to grow to a larger size (Flick et al., 1998). This response requires the cAMP activated protein kinase PKA (Tokiwa et al., 1994). Serine-149 of Whi5 is a close match for a PKA phosphorylation site. Expression of a Ser-149 to Alanine mutant of Whi5 in cells and monitoring for cell cycle delay in response to glucose signaling would give insight as to whether Ser-149 phosphorylation of Whi5 is implicated in the glucose response pathway.

Furthermore, in vitro kinase assays on Whi5 using various purified kinase complexes could identify the kinases responsible for the non-CDK phosphorylations. Using CDK phosphorylation as a positive control, and using Whi5 non-CDK phosphorylation mutants as negative controls, kinases such as PKA could be assessed for their ability to phosphorylate specific Whi5 sites. The casein kinases Cka1/2 could also be assayed in vitro against Whi5, given that several non-CDK sites are in near consensus sequences for that kinase. Once the kinase(s) responsible for the non-CDK phosphorylations of Whi5 are identified, they may give insight into the regulatory function of the non-CDK sites.

In addition to investigation into the function of Whi5 non-CDK sites, the redundant function of Whi5 and Swi6 phosphorylation raises several
questions. We demonstrated that Whi5-12Ala and Whi5-4Ala CDK mutants caused lethality when overexpressed in Swi6-SA4 cells and resulted in a large cell size when expressed at physiologic levels with Swi6-SA4. Of the four mutant Swi6 CDK sites, only Ser-160 has been assigned a function. Ser-160 phosphorylation inhibits nuclear import of Swi6 (Geymonat et al., 2004; Sidorova et al., 1995). It is unknown whether the other three mutated CDK sites in Swi6-SA4 bear any impact in the synergism with Whi5 CDK mutants.

To determine what CDK sites of Swi6 are important for phosphorylation in combination with Whi5, various Swi6 CDK site mutants could be assayed in cells overexpressing Whi5-12Ala. This would demonstrate if all four CDK sites of Swi6 are necessary, if only Ser-160 was necessary, or if the other three CDK sites were necessary for viability in combination with Whi5-12Ala. In addition, Swi4 has 18 putative CDK phosphorylation sites. It is unknown if they are also redundant with Whi5 CDK phosphorylation. Phosphorylation mutants of Swi4 could be tested in combination with Whi5-12Ala and assayed for cell viability. It is possible that phosphorylation of Whi5 and Swi4 is similarly redundant to Whi5 and Swi6 phosphorylation, evoking a model where SBF activation could result from phosphorylation of either Swi6 and Whi5, Swi4 and Whi5, or just Swi6 and Swi4. The requirement for CDK phosphorylation of members of the SBF transcriptional complex to activate transcription remains to be determined.

Finally, further investigation into the mechanism of Whi5 and Swi6 nuclear export is needed. First of all, it remains to be determined what CDK
sites of Whi5 are required for nuclear export, and if nuclear export requires specific CDK sites or a threshold amount of phosphorylation. Our data shows that Whi5-12Ala remains nuclear, corroborating reports that CDK phosphorylation is required for Whi5 nuclear export. A recent study by Skotheim et al. shows that Whi5 with 6 C-terminal CDK sites mutated to alanine is largely nuclear, but is exported to a small extent as cells bud and enter S-phase (Skotheim et al., 2008). This result, in combination with our data, indicates that the C-terminal CDK sites of Whi5 are required for complete nuclear export, but that N-terminal CDK sites retain the ability to signal for Whi5 export. A detailed analysis of nuclear localization of Whi5 phosphorylation mutants is needed to clarify the sites utilized in nuclear export.

In addition to determining the CDK sites of Whi5 required for export, further investigation is needed to understand the role of SWI6 and MSN5 in Whi5 cellular localization. We demonstrated that Whi5 was retained in the nucleus of both swi6Δ and msn5Δ cells, making Whi5 nuclear export Swi6- and Msn5- dependant. Msn5 is a nuclear export factor, but we were unable to detect interaction between Whi5 and Msn5. It is possible that Msn5 is directly responsible for Whi5 export and our methods did not detect the interaction. Because Swi6 nuclear export has been shown to be Msn5-dependant (Queralt and Igual, 2003), it is possible that Msn5 exports Swi6 and Whi5 from the nucleus together. Analysis of Whi5 localization with mutants of Swi6 that cannot bind Whi5 would determine if Whi5-Swi6 binding was a prerequisite for
export. The converse could be determined for Whi5 mutants that do not bind Swi6. Additionally, localization of Swi6 in \textit{whi5}Δ cells would show if Swi6 is similarly dependant on Whi5 for export.

It is important to note that Whi5 shuttled from the nucleus to the cytoplasm normally in Swi6-SA4 cells. Swi6-SA4 is found localized to the nucleus. However, phosphorylation of Swi6 Ser-160 inhibits nuclear import. When that site is mutated, as in Swi6-SA4, nuclear import cannot be inhibited. The basis for Swi6-SA4 nuclear localization is not a failure to be exported, but a continual nuclear import. It is possible that Swi6-SA4 is able to bind Whi5 and support its export to the cytoplasm via Msn5. From there, Whi5 would remain cytoplasmic while Swi6-SA4 would be shuttled back into the nucleus. This model remains to be tested. de Bruin et al show Swi6 and Whi5 bind throughout the cell cycle, outside of G1, implying that cytoplasmic Whi5 binds Swi6 (de Bruin et al., 2004). Testing whether Whi5 binds to Swi6-SA4 outside of G1 would provide evidence to support this model.

In conclusion, further experiments are needed in both yeast and mammalian cells to better comprehend the regulation by phosphorylation of Whi5 and Rb. Rb is a tumor suppressor protein, and Rb pathway members regulating G1 cell cycle progression are mutated in the majority of cancers. Therefore, investigation into Whi5 regulation and determining how it relates to Rb function could provide insight into how tumor cells override established checkpoints and evade cell cycle controls. Further understanding of Whi5 and
Rb function has future therapeutic implications important to cancer prevention and treatment.
REFERENCES


