CDK Activity Antagonizes Whi5, an Inhibitor of G1/S Transcription in Yeast

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Summary

Cyclin-dependent kinase (CDK) activity initiates the eukaryotic cell division cycle by turning on a suite of gene expression in late G1 phase. In metazoans, CDK-dependent phosphorylation of the retinoblastoma tumor suppressor protein (Rb) alleviates repression of E2F and thereby activates G1/S transcription. However, in yeast, an analogous G1 phase target of CDK activity has remained elusive. Here we show that the cell size regulator Whi5 inhibits G1/S transcription and that this inhibition is relieved by CDK-mediated phosphorylation. Deletion of *WHI5* bypasses the requirement for upstream activators of the G1/S transcription factors SBF/MBF and thereby accelerates the G1/S transition. Whi5 is recruited to G1/S promoter elements via its interaction with SBF/MBF in vivo and in vitro. In late G1 phase, CDK-dependent phosphorylation dissociates Whi5 from SBF and drives Whi5 out of the nucleus. Elimination of CDK activity at the end of mitosis allows Whi5 to reenter the nucleus to again repress G1/S transcription. These findings harmonize G1/S control in eukaryotes.

Introduction

Irreversible commitment to a round of cell division typically occurs toward the end of G1 phase, a process called Start in yeast and the restriction point in mammalian cells (Pardee, 1989; Cross, 1995). Prior to Start, cells are sensitive to G1 arrest by mating pheromone and nutrient depletion. Similarly, mammalian cell cycle progression is sensitive to withdrawal of growth factors prior to the restriction point. Events at Start depend on activation of Cdc28, the cyclin-dependent kinase (CDK) responsible for the major cell cycle transitions in budding yeast. Cdc28 is activated in late G1 phase by three G1 cyclins, initially by the upstream G1 cyclin Cln3 and then subsequently by the downstream G1 cyclins Cln1 and Cln2 (Cross, 1995). A primary target of Cln1/2-Cdc28

kinases is the CDK inhibitor Sic1, which binds and inhibits all B-type cyclin (Clb)-Cdc28 complexes (Schwob et al., 1994). Once Sic1 is eliminated, Clb-Cdc28 kinase activity is liberated to initiate DNA replication, spindle maturation, and chromosome segregation (Nasmyth, 1996). At the end of mitosis, Clb-Cdc28 is inactivated to reestablish the permissive G1 state for loading of origins of DNA replication (Nasmyth, 1996; Visintin et al., 1998).

Start depends on activation of a program of G1/S gene expression, which includes the *CLN1/2* and *CLB5/6* genes (Breeden, 1996). G1/S transcription is dictated by two heterodimeric transcription factor complexes called SBF (Swi4/6 Cell Cycle Box [SCB] Binding Factor), composed of Swi4 and Swi6, and MBF (MluI Cell Cycle Box [MCB] Binding Factor), composed of Mbp1 and Swi6. The DNA binding domain of Swi4 recognizes SCB promoter elements in key G1 regulatory genes, while that of its homolog Mbp1 recognizes MCB promoter elements in DNA synthesis and metabolism genes. Although many genes contain both SCB and MCB elements, SBF plays a dominant role at Start, since loss of Swi4 causes a severe cell cycle phenotype, whereas loss of Mbp1 has little phenotypic consequence (Breeden, 1996). Transcriptional activation at G1/S promoters follows a complex ordered series of events first delineated for the developmental and cell cycle-regulated *HO* promoter (Cosma et al., 1999). At *HO*, an initial Swi5 binding event recruits in sequence the Swi/Snf chromatin remodeling complex, the SAGA histone acetylase complex, the SBF complex, the SRB/mediator complex, and finally the CDK-dependent recruitment of Pol II and general transcription factors. Similarly, at the promoters of G1/S-regulated genes such as *CLN2* and *PCL1*, SBF binds and recruits SRB/mediator in early G1 phase, but recruitment of the general transcription machinery does not occur until Cln3-Cdc28 is activated in late G1 phase (Cosma et al., 2001). Though not proven, it is likely that MBF acts in a manner similar to that of SBF.

Intense application of viral genetics and analysis of oncogene and tumor suppressor gene function has lead to considerable understanding of the control of G1/S transcription in mammalian cells (Sherr and McCormick, 2002; Trimarchi and Lees, 2002). In quiescent cells, the retinoblastoma gene product (Rb) and the two other Rb family members p107 and p130, collectively referred to as "pocket proteins," bind to E2F transcription factors and repress transcription through recruitment of histone deacetylase activity (Kaelin, 1999). Initial growth factor stimulus of quiescent cells activates *cyclin D* expression, which results in phosphorylation of Rb by cyclin D-CDK4/6 complexes and liberation of E2F to activate G1/S transcription. Amongst numerous other genes, E2F activates *cyclin E*, which enforces the onset of transcription through additional phosphorylation of Rb. The E2F gene family has diversified through evolution such that family members differentially activate or repress different gene sets, including those required later in the cell cycle, in the DNA damage and checkpoint responses and in chromosome segregation and dynamics (Ren et

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al., 2002; Trimarchi and Lees, 2002). The p16-cyclin D-Rb pathway is disrupted in virtually all cancers and is a critical determinant of growth and proliferation in flies (Neufeld et al., 1998; Sherr and McCormick, 2002). Rb homologs have been identified in all metazoans and in the single-celled green algae *Chlamydomonas reinhardtii* (Kaelin, 1999; Umen and Goodenough, 2001). Despite this conservation, an Rb equivalent has not emerged from extensive genetic and biochemical analysis of the yeast cell cycle.

The timing of transcriptional activation at Start depends heavily on *CLN3*. Cells that lack *CLN3* are large and severely delayed for onset of G1/S transcription, while ectopic induction of *CLN3* in small G1 cells activates transcription and accelerates passage through Start (Tyers et al., 1993; Dirick et al., 1995; Stuart and Wittenberg, 1995). The expression of *CLN3*, *SWI4*, and other genes peaks at the M/G1 boundary. Several other determinants of G1/S transcription have been identified. *BCK2* functions in parallel to *CLN3*, since *cln3 bck2* cells permanently arrest as large unbudded cells (Wijnen and Futcher, 1999). The role of Bck2 is unclear, although overexpression of *BCK2* can activate SBF-dependent transcription in a Cdc28-independent manner (Wijnen and Futcher, 1999). Another factor, Stb1, also assists MBF activation in parallel to Cln3 (Ho et al., 1999; Costanzo et al., 2003). The MAP kinase Slt2 activates a subset of G1/S-regulated cell wall biogenesis genes through its physical association with and phosphorylation of SBF (Baetz et al., 2001). Finally, the pheromone-activated CDK inhibitor Far1 appears to inhibit Cln3 and repress G1/S transcription in pheromone-arrested cells (Jeoung et al., 1998).

Despite much effort, the mechanism whereby Cln3-Cdc28 and other upstream regulators activate SBF/MBF is unclear. Genetic evidence suggests that Swi6 is a critical target of Cln3 (Wijnen et al., 2002). Both Swi4 and Swi6 are readily phosphorylated by upstream kinases, and, yet, mutation of numerous phosphorylation sites in Swi4 and Swi6 has no apparent effect on the timing of G1/S transcription (Sidorova et al., 1995; Wijnen et al., 2002). However, Clb6-Cdc28-mediated phosphorylation of Ser160 partitions Swi6 out of the nucleus from early S phase until late mitosis (Sidorova et al., 1995; Geymonat et al., 2004). Swi6 is also negatively regulated by phosphorylation in response to DNA damage in G1 phase, which delays cells prior to Start (Sidorova and Breeden, 1997). The lack of direct connection between Cln3-Cdc28 and SBF/MBF has led to the suggestion that an unidentified intermediary factor may respond to Cln3-Cdc28 activation (Cosma et al., 2001; Wijnen et al., 2002).

Start does not occur until cells have passed a critical cell size threshold, as modulated by nutrient conditions: in rich medium, cells pass Start at a large critical cell size, while, under conditions of nitrogen or carbon source limitation, cells pass Start at a small cell size (Johnston et al., 1979). Small cell size mutants, referred to as *whiskey* (*whi*) mutants, have proven informative because such mutations by definition accelerate commitment to division. For example, the *WHI1-1* mutation encodes a hypermorphic form of Cln3 (Nash et al., 1988), while the *WHI3* gene encodes an mRNA binding protein that antagonizes *CLN3* activity (Nash et al., 2001b). Recent

systematic analysis of cell size profiles for the entire set of budding yeast deletion mutants has uncovered many new regulators of Start (Jorgensen et al., 2002; Zhang et al., 2002). Amongst these, *WHI5* is one of the most intriguing. Cells that lack *WHI5* grow at a normal rate yet are 30% smaller than wild-type and are resistant to mating pheromone arrest (Jorgensen et al., 2002). Here, we demonstrate that Whi5 binds and inhibits SBF and that CDK-dependent phosphorylation relieves this inhibition. Whi5 thus functions equivalently to the Rb family proteins, a finding that unifies cell cycle control in yeast and metazoans.

Results

Association of Whi5 with Swi4 and Swi6

To interrogate Whi5 function, we sought proteins that physically interact with Whi5. A one-step immunoaffinity purification procedure was used to isolate Whi5[FLAG] complexes from yeast (Ho et al., 2002). Mass spectrometric sequencing of the Whi5-associated proteins yielded 13 Swi4-derived peptides and six Swi6-derived peptides (see Supplemental Figure S1 at http://www.cell.com/cgi/content/full/117/7/899/DC1). We verified the Whi5-SBF interactions by specific detection of both Swi4 and Swi6 in endogenous Whi5[MYC] immune complexes (Figure 1A). The similar recovery efficiencies of each of the three proteins in the Whi5[MYC], Swi4, and Swi6 immunoprecipitates suggested that Whi5 may avidly bind SBF in vivo. Whi5 was also detected in Mbp1 and Stb1 immune complexes (Supplemental Figure S1). Whi5 associated with Swi4 and Swi6 in a mutually dependent manner, even when each gene was expressed from a heterologous promoter (Figure 1B). To determine if Whi5 binds directly to SBF, we produced and purified a soluble Swi4[FLAG]-Swi6 complex from baculovirus-infected insect cells, which was then assembled in solution with Whi5[GST] produced in bacteria. Capture of limiting amounts of recombinant material from the mixture revealed a stoichiometric Whi5-Swi4-Swi6 complex (Figure 1C). Whi5 thus interacts directly with SBF.

Isolation of Whi5 as an Inhibitor of G1/S Transcription

In parallel, we performed a systematic genetic screen for inhibitors of SBF-dependent transcription. A *cln3Δ his3Δ SCB::HIS3* strain, which is a histidine auxotroph because of the SBF defect caused by the absence of *CLN3*, was mated to 4812 viable gene deletion strains using synthetic genetic array (SGA) methodology (Tong et al., 2001). After haploid selection, deletion mutants that express sufficient levels of *HIS3* to confer viability were isolated by pinning the array onto medium containing 30 mM 3-aminotriazole (3′-AT), a competitive inhibitor of the imidazoleglycerol-phosphate dehydratase enzyme encoded by *HIS3*. Two mutants grew vigorously: one in which the *WHI5* ORF (*YOR083w*) is deleted and one in which an overlapping putative ORF, *YOR082w*, is deleted (Figure 2A). A number of other weakly 3′-AT-resistant deletion strains were recovered in the screen, but these were not analyzed further (see Supplemental Table S1 at *Cell* web site). Reactivation of both SBF- and MBF-dependent transcription by the
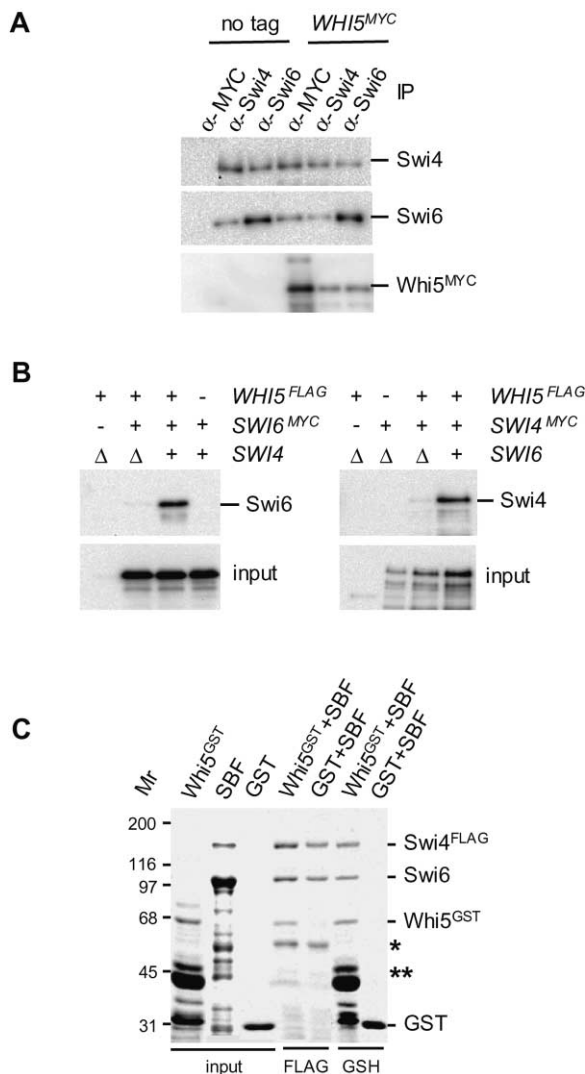
A

B

C

Figure 1. Physical Interactions of Whi5 with SBF and MBF

(A) Coimmunoprecipitation of Whi5 with Swi4 and Swi6. Anti-Myc immune precipitates of lysates from strains bearing either *WHI5* or *WHI5^MYC* at the chromosomal locus (Y4741, MTY3248) were probed with 9E10 anti-MYC and affinity-purified anti-Swi4 and anti-Swi6 antibodies.

(B) Codependence of Whi5 interaction with Swi4 and Swi6. Anti-FLAG immune precipitates of lysates from the indicated strains expressing *WHI5^FLAG* and either *SWI4^MYC* or *SWI6^MYC* were probed with 9E10 anti-MYC antibody.

(C) Whi5 interacts directly with SBF. Purified recombinant Swi4^FLAG-Swi6 complex produced in insect cells was incubated in solution with an approximately stoichiometric amount of Whi5^GST or GST alone produced in bacteria (100% of inputs are shown), captured onto either anti-FLAG or glutathione (GSH) resin, washed, and bound proteins visualized with Coomassie blue stain. Asterisk indicates IgG heavy chain, and double asterisk indicates a Whi5^GST degradation product that is nonfunctional for SBF interaction.

whi5Δ mutation was confirmed in quantitative *lacZ* reporter assays (Figure 2B).

**Genetic Interactions of *WHI5*
with the Start Machinery**

To test if *WHI5* was a dose-dependent inhibitor of Start, we determined the effect of *WHI5* overexpression. In-

duction of a *GAL1-WHI5* construct in an asynchronous culture of wild-type cells caused a modest accumulation of unbudded cells and depletion of the S-phase fraction (Figure 2C). Despite this G1 delay, overexpression of *WHI5* was not lethal, suggesting that the normal processes that activate Start are able to overcome excess Whi5 (Figure 2D). We therefore tested whether a strain genetically compromised for Start was susceptible to increased *WHI5* dosage. Induction of *GAL1-WHI5* in a *cln3Δ* strain caused a uniform arrest as large unbudded cells with 1N DNA content (Figures 2C and 2D). Expression of *GAL1-WHI5* was also lethal in *swi6Δ* and *cdc28-4* strains and severely retarded growth of a *cln1Δ cln2Δ* strain but had little effect on a *swi4Δ* or a *bck2Δ* strain (Figure 2D and data not shown).

Cells that lack both known upstream activators of G1/S transcription, *CLN3* and *BCK2*, permanently arrest at Start (Wijnen and Futcher, 1999). To test whether deletion of *WHI5* might overcome this defect, we compared the ability of *cln3Δ bck2Δ* and *cln3Δ bck2Δ whi5Δ* strains that conditionally express a *GAL1-CLN3* construct to grow on glucose medium, which represses the *GAL1* promoter. Quite remarkably, the *cln3Δ bck2Δ whi5Δ* triple mutant was viable and grew as vigorously as a wild-type strain (Figure 2E).

***WHI5* Size Epistasis**

We characterized genetic interactions between *WHI5* and known Start regulators by size epistasis (Figure 3A). The large cell size of a *swi6Δ* mutant is epistatic to the small cell size of a *whi5Δ* mutant, as shown previously for the *swi4Δ whi5Δ* double mutant (Jorgensen et al., 2002). Conversely, the small size of *whi5Δ* mutant was partially epistatic to the large size of a *cln3Δ* mutant and equal to the small size phenotype conferred by a *CLN3-1* mutation. The pathway may thus be ordered *CLN3-WHI5-SWI4/SWI6* with respect to cell size. However, because overexpression of *CLN3-1* was able to drive *whi5Δ* cells to an even smaller size, we infer the existence of at least one additional redundant pathway that is susceptible to Cln3-Cdc28. A *bck2Δ* mutation causes a less severe size defect than *cln3Δ*, such that its interaction with *whi5Δ* appeared additive. Overexpression of *BCK2* resulted in an extremely small cell size that was not affected by *WHI5* status, consistent with the ability of *BCK2* to bypass the requirement for Cdc28 at Start (Wijnen and Futcher, 1999). To examine the role of *WHI5* in nutrient regulation of the size threshold, we determined the size of wild-type and *whi5Δ* strains grown on different carbon sources. Cell size was concordantly reduced on poor carbon sources in wild-type and *whi5Δ* strains (Figure 3B), as has been reported for *CLN3-1* strains (Nash et al., 1988). Whi5 therefore does not mediate regulation of cell size by carbon source.

**Whi5 Affects Onset but Not Periodicity
of G1/S Transcription**

To examine the influence of Whi5 on the timing of G1/S transcription, early G1 phase daughter cells from wild-type and *whi5Δ* cultures were isolated by centrifugal elutriation and released into fresh medium. In order to obtain sufficient quantities of small G1 phase *whi5Δ* cells, both cultures were grown to near stationary phase
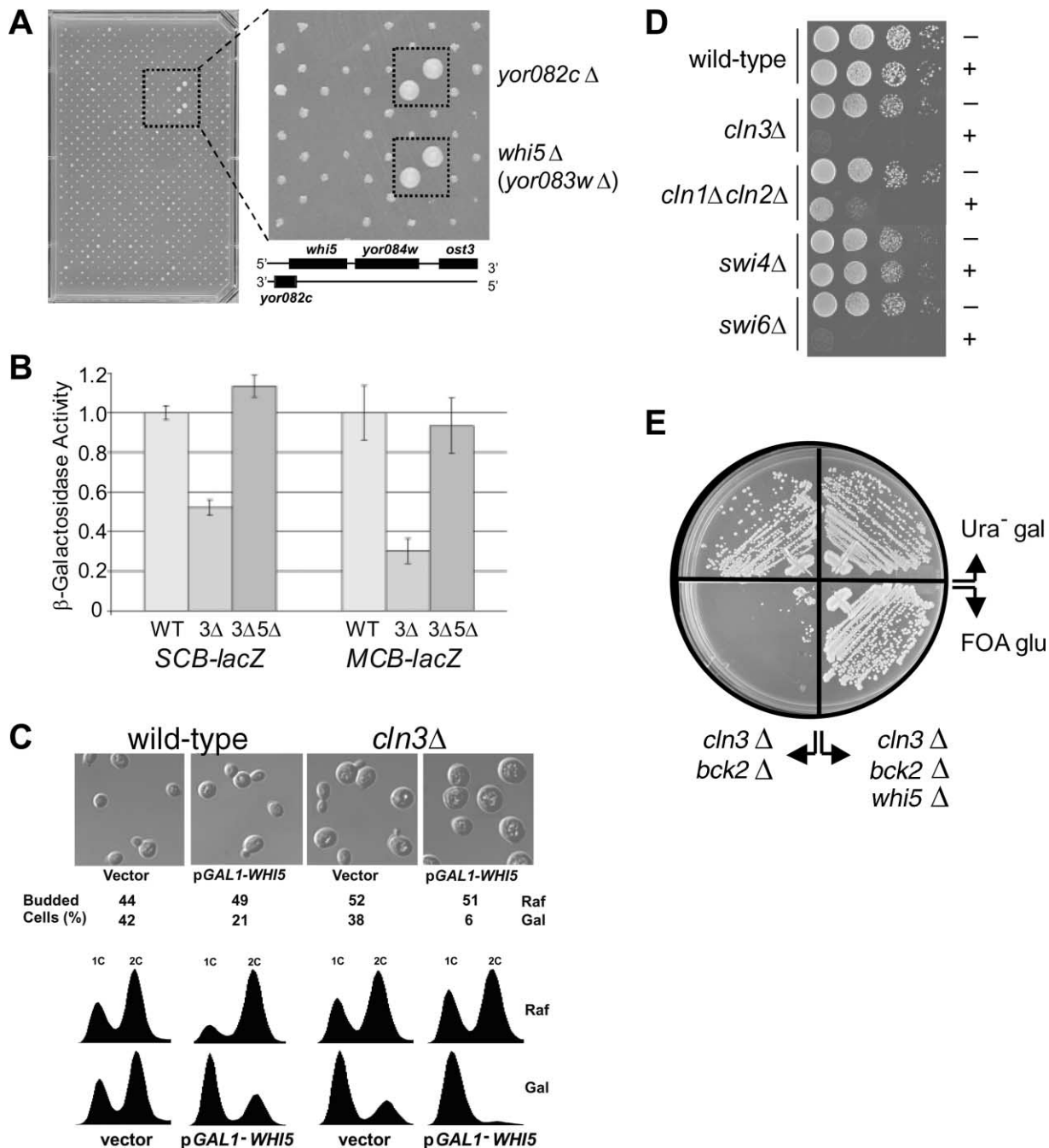
Figure 2. Genetic Interaction of *WHI5* with the Start Machinery

(A) Isolation of *whi5Δ* in a systematic genetic screen for inhibitors of SCB-dependent transcription. A *cln3Δ* strain containing an *SCB-HIS3* reporter (BY2054) was mated to 4812 haploid strains using SGA methodology. Double mutant haploids were selected and assayed for growth on medium containing 30 mM 3-aminotriazole (3′-AT).

(B) Deletion of *WHI5* overcomes defective SCB- and MCB-dependent transcription in a *cln3Δ* strain. Lysates from the indicated strains (BY2700, BY2701, BY2702) bearing *SCB::lacZ* or *MCB::lacZ* reporter constructs were assayed for β-galactosidase activity. Values are mean ± SEM from triplicate determinations.

(C) Overexpression of *WHI5* in the absence of *CLN3* causes a G1 phase arrest. Wild-type (Y4741) and *cln3Δ* (BY655) strains bearing either vector (p425 GAL1) or *GAL1-WHI5* (pMT3586) plasmids were grown in raffinose medium and then induced in galactose medium for 3 hr, imaged by DIC microscopy, and scored for bud index and DNA content.

(D) Overexpression of *WHI5* is lethal in strains compromised for Start. Isogenic wild-type (Y4741), *cln3Δ* (BY655), *cln1Δ cln2Δ* (BY438), *swi4Δ* (consortium), and *swi6Δ* (consortium) strains bearing either *GAL1-WHI5^FLAG* (+, pMT3586) or empty vector (−, p425) were spotted in serial 10-fold dilutions on galactose medium and incubated for 48 hr at 30°C.

(E) Deletion of *WHI5* restores viability to a *cln3Δ bck2Δ* strain. A *whi5Δ/WHI5 cln3Δ/CLN3 bck2Δ/BCK2* diploid strain bearing a *GAL1-CLN3-URA3* plasmid (pMT41) was generated by crossing two haploid strains (MTY2646 with pMT41 to MTY2125) and subsequent dissection on galactose medium to induce *GAL1-CLN3* expression. *cln3Δ bck2Δ* and *whi5Δ cln3Δ bck2Δ* spore clones were streaked on either Ura⁻ galactose or 5-FOA glucose medium and incubated at 30°C for 48 hr.
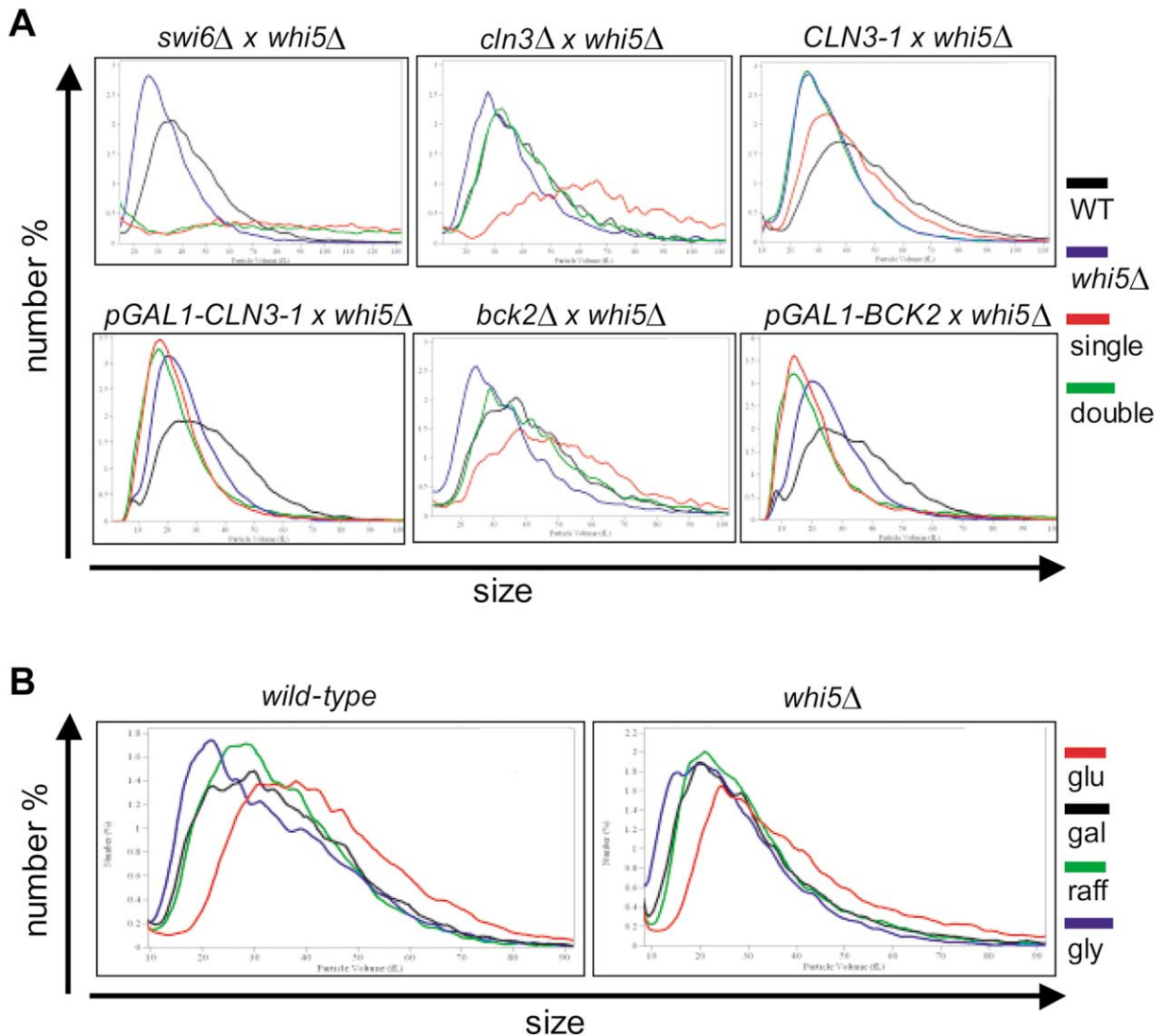
Figure 3. Modulation of Cell Size by Whi5

(A) Size epistasis between *whi5Δ* and other Start mutations. Isogenic *swi6Δ* (MTY2574), *cln3Δ* (MTY2125), *CLN3-1^HA3* (MTY2208), *GAL1-CLN3-1* (MTY2128), *bck2Δ* (MTY2299), and *GAL1-BCK2* (MTY3252) strains were crossed to *whi5Δ* (MTY3249) and sets of tetratype spore clones analyzed for cell size distribution.

(B) Whi5 is not required for alteration of critical cell size by carbon source. Wild-type (Y4741) and *whi5Δ* (MTY3249) strains were sized after growth to early log phase in rich medium containing the indicated carbon sources.

prior to elutriation. In wild-type cells, a peak in transcription of the SBF-regulated gene *CLN2* and the MBF-regulated gene *RNR1* was observed at 27 fl, coincident with the onset of bud emergence and DNA replication. In contrast, *CLN2* and *RNR1* expression was evident even in the initial 11 fl culture of the *whi5Δ* strain, with a peak at 17 fl, consistent with the earlier onset of bud emergence and DNA replication (Figure 4A). Similar results were obtained when *CLN2* and *RNR1* expression was examined in a series of size fractions obtained by elutriation of a log phase *whi5Δ* culture (Supplemental Figure S2).

Accelerated G1/S transcription might arise as a consequence of complete derepression of SBF- and MBF-dependent genes. To test this possibility, we examined the periodicity of *CLN2* and *RNR1* expression in wild-

type and *whi5Δ* cultures released synchronously into the cell cycle from a G1 phase arrest imposed by mating pheromone. Although a *whi5Δ* strain is more resistant to mating pheromone than wild-type (Jorgensen et al., 2002), the strain arrested uniformly in response to high concentrations of pheromone (Figure 4B). After release from the block, the *whi5Δ* culture exhibited only a slight acceleration of *CLN2* expression compared to wild-type cells. The similar timing of G1/S transcription in wild-type and *whi5Δ* strains in this context was attributable to continued growth in the pheromone block, such that both wild-type and *whi5Δ* strains exceed the size threshold at the point of release. That is, elimination of Whi5 was not rate limiting in the wild-type strain under this circumstance. Significantly, *CLN2* and *RNR1* exhibited the same periodic expression in the *whi5Δ* strain as in
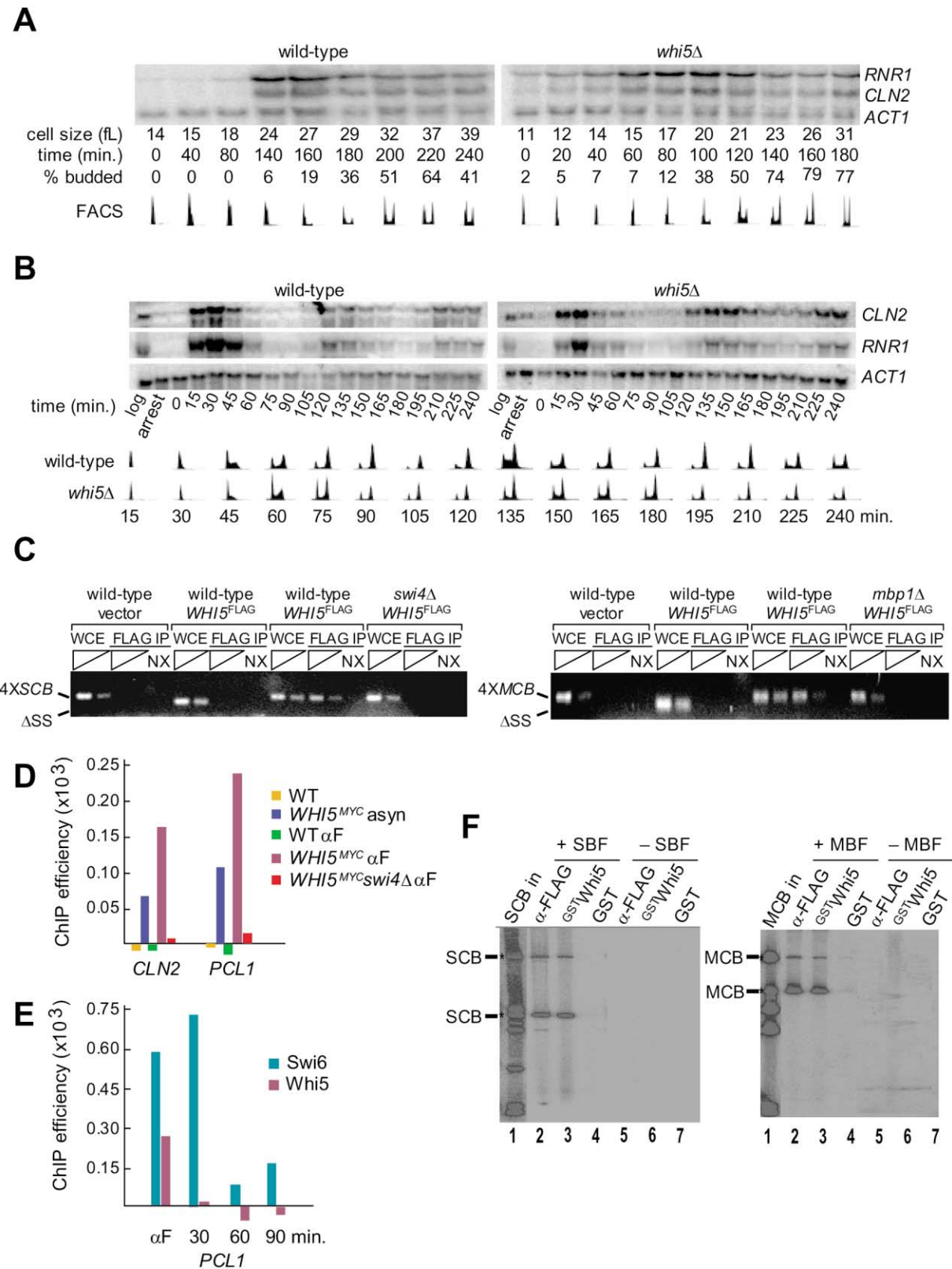
Figure 4. Whi5 Regulates SBF and MBF-Dependent Genes

(A) Deletion of *WHI5* accelerates expression of G1/S mRNAs in G1 cells isolated by centrifugal elutriation. Small G1 phase cells from wild-type (Y4741) and *whi5Δ* (MTY3249) strains were isolated by centrifugal elutriation of 2 liter of culture grown to overnight saturation in raffinose medium. The smallest size fractions obtained were inoculated into glucose medium and assessed for *CLN2*, *RNR1*, and *ACT1* mRNA; cell size; bud index; and DNA content at the indicated time points.

a wild-type strain. The mechanisms that normally restrict expression to the G1/S window, namely Clb2-Cdc28 activity, therefore do not depend on Whi5 (Amon et al., 1993; Koch et al., 1996). Rather, Whi5 restrains the onset of G1/S transcription until Cln3-Cdc28 activity is developed at the appropriate cell size.

Recruitment of Whi5 to SBF and MBF-Regulated Promoters

As SBF is bound to SCB-regulated promoters in an inactive state in early G1 phase cells (Harrington and Andrews, 1996; Koch et al., 1996; Cosma et al., 1999), it seemed likely that Whi5 would bind to promoter DNA indirectly. To test this model, we examined the interaction between Whi5 and a synthetic promoter that contains four copies of the consensus SCB element (CAC GAAA) by chromatin immunoprecipitation (Figure 4C). To facilitate detection, the SCB promoter was borne on a high-copy plasmid, and a $WHI5^{FLAG}$ allele was overexpressed from the $GAL1$ promoter. An otherwise identical promoter construct lacking SCB elements, called ΔSS, was used as a control. The SCB-containing promoter but not the ΔSS promoter was recovered in Whi5^{FLAG}-associated chromatin complexes in a manner that depended on treatment with formaldehyde crosslinker. Whi5^{FLAG} failed to capture SCB promoter DNA in a $swi4\Delta$ strain, indicating a requirement for physiological levels of Swi4. Identical results were obtained for capture of an analogous synthetic promoter that contained four copies of a consensus MCB element (ACGCGT), in this case in an $MBP1$-dependent manner (Figure 4C).

We assessed the occupancy of endogenous SBF-regulated promoters by endogenous levels of Whi5^{MYC} using real-time (RT) PCR to detect promoter fragments in chromatin immunoprecipitates. Both $CLN2$ and $PCL1$ promoter sequences were specifically detected in association with Whi5^{MYC} complexes (Figure 4D). These interactions increased in cells arrested in G1 phase by pheromone and were dependent on $SWI4$. Cell cycle regulation of Whi5-promoter DNA interactions was demonstrated by release of cultures from a pheromone arrest. Within 30 min of release, Whi5 was no longer detected at the $PCL1$ promoter, whereas Swi6 was retained (Figure 4E). At later time points, Swi6 also departed from promoter DNA, as expected from the known nuclear export of Swi6 once G1/S transcription has sub-

sided (Sidorova et al., 1995; Geymonat et al., 2004). As primer design constraints for RT-PCR precluded detection of the few genes whose expression depends exclusively on MBF in Whi5 chromatin complexes, the role of the Whi5-MBF interaction under strictly physiological circumstances remains to be established.

To demonstrate that Whi5 is bridged to promoter DNA solely by SBF or MBF, we recapitulated the Whi5-SBF/MBF-DNA interactions in a purified system. Whi5^{GST} produced in bacteria was incubated with a digested [^{32}P]-labeled plasmid that contained SCB promoter elements, in the presence or absence of purified SBF produced in insect cells (Figure 4F). As expected, capture of Swi4^{FLAG}-Swi6 on anti-FLAG resin specifically recovered restriction fragments that contained the SCB elements, while capture of Whi5^{GST} alone on GST resin did not recover any DNA fragments. However, capture of Whi5^{GST} in the presence of SBF fully recapitulated the pattern of fragments recovered with SBF. To determine if Whi5 also binds to MBF, an analogous experiment was performed with recombinant MBF and a MCB-containing plasmid. Whi5 associated with promoter fragments in the presence but not in the absence of purified MBF (Figure 4F). Whi5 thus associates indirectly with promoter DNA via its interaction with SBF or MBF.

Cell Cycle-Regulated Nuclear Localization of Whi5

One model we entertained for the repression of G1/S transcription was that Whi5 abundance might be periodic in a similar manner to the CDK inhibitor Sic1 (Schwob et al., 1994; Nash et al., 2001a). Whi5^{MYC} produced from the endogenous locus was readily detected in cells arrested in G1 phase by mating pheromone, by nutrient depletion, or by overexpression of a stable dominant allele of $SIC1$ called $SIC1^{0P}$ (Nash et al., 2001a) that lacks all CDK phosphorylation sites (Figure 5A). However, Whi5^{MYC} was equally abundant in cells arrested in early S phase with hydroxyurea, in mitosis with nocodazole, and in telophase with a $cdc15-2$ mutation. Furthermore, $WHI5$ mRNA is not cell cycle regulated (Spellman et al., 1998).

Many transcription factors are controlled at the level of nuclear localization, including the cell cycle-regulated factors Swi5 and Swi6 (Moll et al., 1991; Sidorova et al., 1995). We therefore determined the localization of a functional Whi5^{GFP} fusion protein in cells arrested at dif-

(B) $WHI5$ does not affect periodicity of G1/S mRNA mRNA expression. Wild-type (Y4741) and $whi5\Delta$ (consortium) strains were arrested in G1 phase with α factor and then synchronously released into the cell cycle. $CLN2$, $RNR1$, and $ACT1$ mRNA and DNA content were assessed at the indicated time points.
(C) Swi4- and Mbp1-dependent interaction of Whi5 with synthetic SCB and MCB promoters. Anti-FLAG chromatin immunoprecipitations from either wild-type (Y4741), $swi4\Delta$ (consortium), or $mbp1\Delta$ (consortium) strains expressing a $GAL1$-$WHI5^{FLAG}$ construct (pMT3586) or not and bearing either synthetic $SCB::lacZ$ (pBA251), $MCB::lacZ$ (pBA487), or control (ΔSS, pLGΔSS) plasmids were analyzed for the presence of promoter elements by PCR. Input extract for each immunoprecipitation was assessed with the same PCR protocol. Reactions were performed on cells treated with or without (NX lanes) formaldehyde crosslinker.
(D) Location of Whi5 on endogenous SBF-dependent promoters. Anti-MYC chromatin immunoprecipitations from the indicated strains were analyzed for $CLN2$ and $PCL1$ promoter sequences by quantitative RT-PCR. Where indicated, strains were exposed to 5 μM α factor for 2 hr prior to treatment with crosslinker.
(E) Cell cycle-regulated interaction of Whi5 and Swi6 with an SBF-dependent promoter. A strain expressing WHI5^{MYC} from the endogenous locus was arrested with 5 μM α factor for 2 hr, released from the arrest, and treated with crosslinker at the indicated time points. Anti-MYC and anti-Swi6 chromatin immunoprecipitations were analyzed for $PCL1$ promoter sequences by RT-PCR as in (D).
(F) Capture of SCB and MCB promoter elements by recombinant Whi5-SBF and Whi5-MBF complexes. Recombinant complexes were incubated with HinfI-digested ^{32}P-labeled plasmids containing SCB or MCB promoter elements, and protein-DNA complexes were captured with the indicated resins and resolved by gel electrophoresis. DNA fragments containing SCB and MCB elements are indicated.
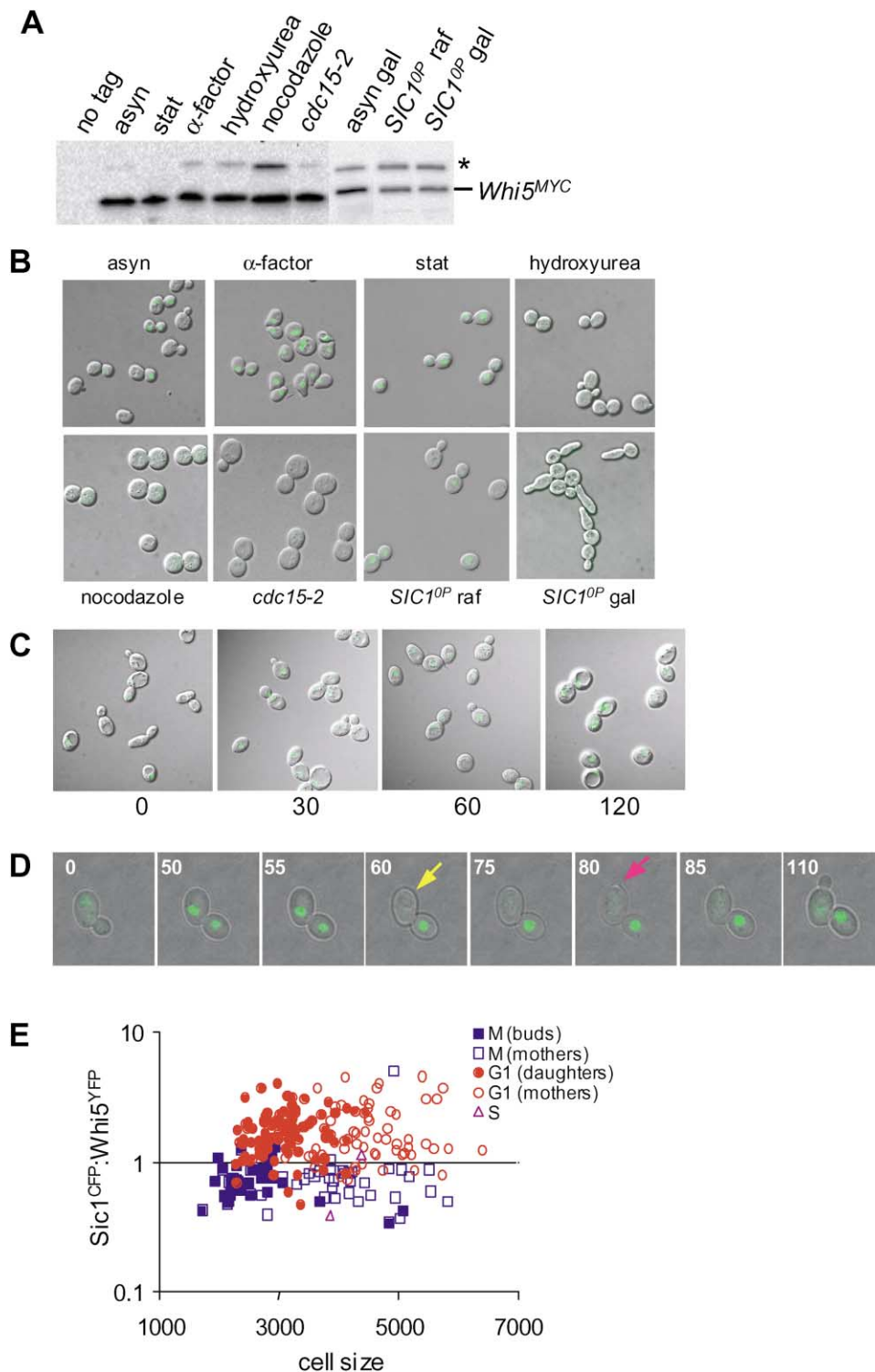
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Figure 5. Whi5 Nuclear Localization

(A) Whi5 abundance is not cell cycle-regulated. Cultures of strains expressing *WHI5^MYC* (MTY3248) at the endogenous locus were arrested in G1 phase by α factor, by growth overnight into stationary phase, or by overexpression of a dominant nonphosphorylatable allele of *SIC1* (pMT2728) for 3 hr, in S phase with hydroxyurea, in metaphase with nocodazole, and in telophase by shift of a *cdc15-2* temperature-sensitive mutant (MTY3253) to 37°C for 3 hr. Whi5^MYC abundance was assessed by immunoblot. Asterisk indicates a crossreactive species of unknown nature that appears specific to *WHI5^MYC* strains.

(B) Whi5 is nuclear localized only in G1 phase cells. Cultures of strains expressing *WHI5^GFP* (MTY3256, MTY3254) at the endogenous locus were arrested as in (A) and assessed for Whi5^GFP fluorescence. Representative fields are shown.

(C) Whi5 relocalizes to the nucleus of budded cells upon inactivation of Cdc28. A *cdc28-4* temperature-sensitive strain expressing *WHI5^GFP* at the endogenous locus (MTY3255) was shifted to 37°C for the indicated times in minutes. Representative fields are shown.

ferent stages of the cell cycle, as above (Figure 5B). In asynchronous cultures, Whi5^{GFP} was only present in the nucleus of late mitotic and G1 phase cells and never in small budded cells. Whi5^{GFP} was readily detected in the nucleus of cells arrested in G1 phase by either phero- mone or nutrient depletion. Both of these G1 states lack CDK activity. In contrast, Whi5^{GFP} was completely excluded from the nucleus in cells arrested in G1 phase by overexpression of SIC1^{0P}. The multibudded pheno- type that characterizes this arrest is due to high levels of Cln-Cdc28 activity (Schwob et al., 1994; Verma et al., 1997). Similarly, Whi5^{GFP} was excluded from the nucleus in hydroxyurea, nocodazole, and cdc15-2 arrests, all of which represent high CDK activity states. We tested the notion that CDK activity drives Whi5 from the nucleus by examining Whi5^{GFP} localization upon conditional inac- tivation of Cdc28 in a cdc28-4 strain. Upon shift to the restrictive temperature, Whi5 became localized to the nucleus in all cells within 30 min, regardless of cell cycle position (Figure 5C).

To verify cell cycle-dependent nuclear relocalization of Whi5 in an unperturbed state, we followed Whi5^{GFP} subcellular location in a single dividing cell (Figure 5D). Whi5 first appeared in both the mother and daughter cells in late mitosis, presumably after activation of the mitotic exit network had eliminated CDK activity. Whi5 was exported from the mother cell nucleus approxi- mately 20 min before bud emergence. A similar differen- tial in the timing of Whi5 export and bud emergence occurred in the daughter cell (data not shown). The tim- ing of Whi5 import/export was benchmarked against Sic1, which appears in the nucleus shortly after mitotic exit and is then degraded at Start (Nash et al., 2001a). A strain bearing integrated WHI5^{YFP} and SIC1^{CFP} alleles at the endogenous loci was highly enriched for Whi5^{YFP} over Sic1^{CFP} nuclear signal in cells in late mitosis, while late G1 phase cells were highly depleted for Whi5^{YFP} relative to Sic1^{CFP} nuclear signal (Figure 5E). Whi5 nu- clear localization thus precisely mirrors the low CDK state.

Whi5 Is a Substrate of Various Cyclin-Cdc28 Complexes

The physical interaction of kinases and their substrates can often be detected directly by copurification of the proteins or indirectly by association of kinase activity with the substrate. The low abundance of Cln3-Cdc28 complexes renders such experiments technically diffi- cult (Tyers et al., 1992). Trace amounts of Whi5 could be found in association with endogenous levels of Cln3-1 and with overexpressed Cln2 or Cdc28 (data not shown). To determine if Whi5 was physically associated with Cdc28 activity, we incubated Whi5^{FLAG} immune complexes isolated from wild-type and cdc28-4 strains with [^{32}P]-γ-ATP and resolved the reaction products by

SDS-PAGE. Whi5^{FLAG} recovered a robust autophosphor- ylation activity from cells that was partially CDC28 de- pendent (Figure 6A).

Whi5 appeared to be indiscriminately excluded from the nucleus by all forms of CDK activity. However, be- cause Cln3 is expressed before all other cyclins, its activity would dictate the initial export of Whi5 (Tyers et al., 1993; McInerny et al., 1997). To further address the issue of cyclin specificity, we compared the relative rates of phosphorylation of Whi5^{GST} and Sic1^{GST} by Cln2- Cdc28, Cln3-Cdc28, and Clb5-Cdc28 complexes (Figure 6B). Both substrates were phosphorylated with the same relative efficiency by the Cln2- and Cln3-Cdc28 kinases over a range of substrate concentrations. Clb5-Cdc28 was partially inhibited by high concentrations of Sic1^{GST} as expected but nevertheless phosphorylated Whi5^{GST} in a linear manner, just as observed for the Cln-Cdc28 kinases. Most of the observed [^{32}P] incorporation was at CDK sites, since, in control reactions, neither Sic1^{0PGST} nor a version of Whi5 that lacks all 12 Ser/Thr-Pro mini- mal CDK consensus sites (Whi5^{12AGST}) was phosphory- lated to any appreciable extent. Whi5 is thus a promiscu- ous substrate of cyclin-CDK activity in vitro.

Phosphorylation Controls Whi5 Nuclear Localization and the Whi5-SBF Interaction

To show that nuclear exclusion was due to phosphoryla- tion of Whi5 itself as opposed to indirect effects on nuclear transport (Makhnevych et al., 2003), we intro- duced an allele that lacks all six C-terminal CDK sites (WHI5^{6A}) at the endogenous WHI5 locus. This region, including the six CDK sites, is conserved in a similar protein from the distantly related yeast Ashbya gossypii (Dietrich et al., 2004). The cell cycle dependence of Whi5 localization was dramatically altered by the loss of C-terminal CDK phosphorylation sites as Whi5^{6A}-GFP was localized to the nucleus over the entire cell cycle (Figures 6C and 6D). However, WHI5^{6A} had no overt ef- fect on cell viability or progression through Start.

As constitutive Whi5 nuclear localization evidently does not preclude SBF activation, additional mecha- nisms must inactivate Whi5. We therefore determined whether CDK kinase activity was able to modulate the composition of recombinant Whi5-SBF complexes. For this purpose, soluble Whi5^{GST} produced in bacteria was pre bound to insect cell-produced Swi4^{FLAG}-Swi6 com- plex on anti-FLAG resin, incubated with Cln2-Cdc28 in the presence of ATP, and separated into released and bound fractions (Figure 6E). Phosphorylation caused ap- proximately 50% of the SBF bound Whi5 to be released. The partial release may reflect incomplete phosphoryla- tion of one or more critical sites on Whi5, Swi4, or Swi6. As controls, incubation in the presence of either ADP or the nonhydrolyzable ATP analog AMP-PNP caused only a background level of Whi5 to dissociate, due in

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(D) Whi5 is exported from the nucleus prior to Start. A single cell from a strain bearing a WHI5^{GFP} fusion integrated at the endogenous locus (MTY3001) was assessed for GFP fluorescence at the indicated times in minutes. The yellow arrow indicates disappearance of Whi5^{GFP} in the mother cell, while the pink arrow indicates appearance of the bud.
(E) Whi5 nuclear entry and exit precede accumulation and destruction of Sic1. A strain bearing WHI5^{YFP} and SIC1^{CFP} at the endogenous loci (MTY3176) was assessed for nuclear YFP and CFP fluorescence by quantitative microscopy. The ratio of CFP:YFP signal was plotted for mother and daughter cells in late mitosis or G1 phase, as assessed by morphology.
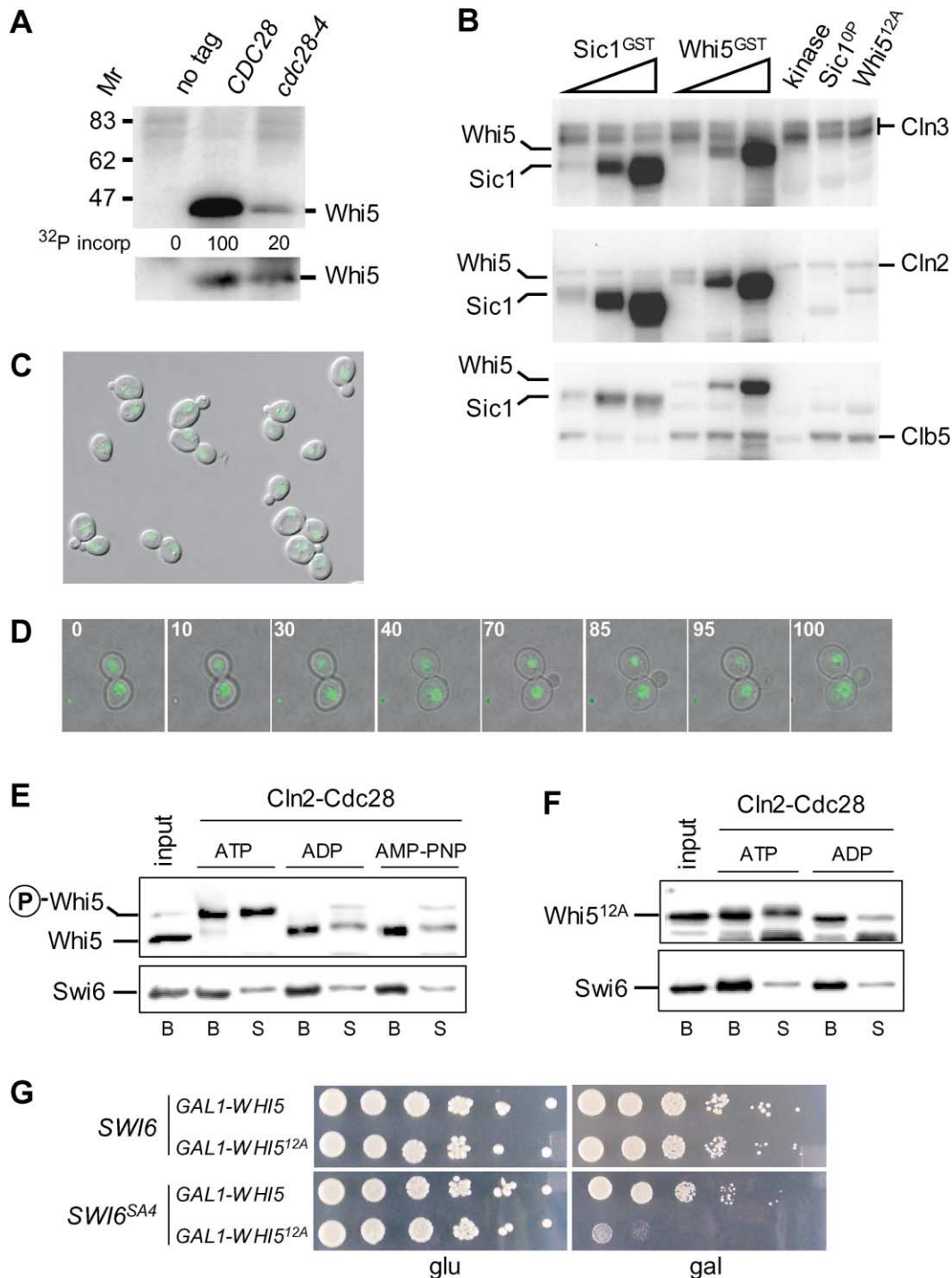
Figure 6. Regulation of Whi5 by CDK-Dependent Phosphorylation

(A) Association of Whi5 with a Cdc28-dependent kinase activity. Wild-type (K699) or *cdc28-4* (K1989a) strains bearing a *GAL1-WHI5^FLAG* plasmid (pMT3586) or control vector (pMT3164) were induced on galactose medium for 3 hr. Whi5 complexes were recovered on anti-FLAG resin, incubated in kinase buffer with [^{32}P]-γ-ATP, and resolved by SDS-PAGE. Captured Whi5 protein was detected with anti-FLAG antibody.

(B) Phosphorylation of Whi5 by Cdc28 kinases in vitro. The indicated amounts of recombinant Whi5^GST and Sic1^GST were phosphorylated in the presence of [^{32}P]-γ-ATP with recombinant Cln3-Cdc28 (top), Cln2-Cdc28 (middle), or Clb5-Cdc28 (bottom) kinases.

(C) Elimination of consensus CDK phosphorylation sites prevents nuclear export of Whi5. An asynchronous culture of a strain bearing a *WHI5^6A-GFP* allele integrated at the endogenous locus (MTY3004) was assessed for GFP fluorescence. A representative field is shown.

(D) Cell cycle-regulated nuclear export of Whi5 depends on CDK phosphorylation sites. A single cell from a strain carrying a *WHI5^6A-GFP* fusion allele (MTY3004) was assessed for GFP fluorescence at the indicated times in minutes.

(E) Phosphorylation dissociates the Whi5-SBF complex. A preassembled recombinant Whi5-Swi4^FLAG-Swi6 complex bound to anti-FLAG resin was incubated with Cln2-Cdc28 kinase in the presence of either ATP, ADP, or AMP-PNP. After washing, proteins in the bound and supernatant fractions were identified by immunoblot. All Swi4^FLAG signal was retained in the bound fraction (data not shown).

part to release of a small fraction of Swi6 from the FLAG resin. The released Swi6 migrated more slowly than the remaining bound Swi6, suggesting that phosphorylated Swi6 may be preferentially released from Swi4. To determine whether phosphorylation of Whi5 was necessary for CDK-mediated disassembly of the Whi5-SBF complex, we repeated the same experiment with nonphosphorylatable Whi5[12AGST]. Cln2-Cdc28 was indeed still able to dissociate a substantial fraction of Whi5[12A] from SBF (Figure 6F). In toto, these results implicate CDK-dependent phosphorylation in control of both Whi5 nuclear localization and Whi5-SBF interactions.

To test the potential role of redundant phosphorylation on Whi5 and SBF or MBF, we introduced a plasmid expressing *WHI5[12A]* from the inducible *GAL1* promoter into a strain harboring a version of Swi6 that lacks four CDK phosphorylation sites (Sidorova et al., 1995). As in the *WHI5[6A]* mutant, or strains that overexpress wild-type *WHI5*, overexpression of the *WHI5[12A]* mutant allele in wild-type cells caused a minimal phenotype. However, coexpression of both *WHI5[12A]* and *SWI6[SA4]* mutant alleles caused a dramatic growth defect (Figure 6G). Taken together with the inferred redundancy of Whi5 and SBF phosphorylation in vitro, the genetic interaction between these *WHI5* and *SWI6* alleles suggests that supernumerary phosphorylation events liberate G1 transcription factor complexes from Whi5.

Discussion

Whi5 Inhibits SBF/MBF

The means by which Cln3-Cdc28 activates G1/S transcription in yeast has remained a puzzling gap in the understanding of Start. A substantial body of evidence places Whi5 between Cln3-Cdc28 activity and the downstream transcription factors: (1) Whi5 physically associates with Swi4, Mbp1, and Swi6; (2) deletion of *WHI5* overcomes the SCB- and MCB-transcription defects of a *cln3Δ* strain and bypasses the requirement for *CLN3* in the absence of *BCK2*; (3) *WHI5* is genetically downstream of *CLN3* and upstream of *SWI4* and *SWI6* with respect to size epistasis; (4) G1/S transcription is accelerated in the absence of *WHI5*; (5) Whi5 binds to SCB and MCB promoter elements in vivo and in vitro and to endogenous SBF-regulated promoters; (6) Whi5 is associated with a *CDC28*-dependent kinase activity in vivo and is phosphorylated by Cdc28 kinases in vitro; and (7) CDK-dependent phosphorylation dissociates the Whi5-SBF complex and triggers Whi5 nuclear export. Whi5 therefore links G1 phase CDK activity to the broad transcriptional program that accompanies commitment to division. In the scheme of stepwise promoter recruitment elucidated by Cosma and Nasmyth (Cosma et al., 1999, 2001), Whi5 resides at the CDK dependent step that enables SBF to recruit the general transcription
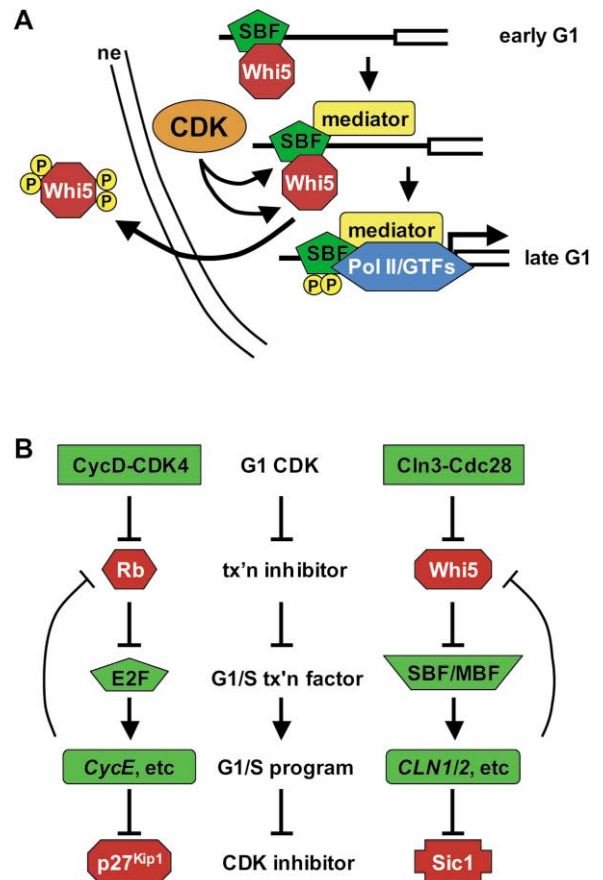

Figure 7. Summary of Whi5 Action and Regulation

(A) CDK-dependent phosphorylation disassembles the Whi5-SBF complex and leads to nuclear export of Whi5, thereby allowing SBF to recruit RNA PolII and general transcription factors (GTFs) to the promoter. MBF is likely regulated in the same fashion.
(B) Genetic equivalence at each step in the Whi5 and Rb pathways that control G1/S transcription in yeast and mammalian cells, respectively.

machinery just prior to transcriptional activation (Figure 7A).

Previous work has hinted at the existence of a negative regulator of G1/S transcription that targets Swi6 (Breeden, 1996; Cosma et al., 2001; Wijnen et al., 2002). Moreover, a truncated form of Swi4, called Swi4[t], bypasses the need for Swi6 and Cdc28 in transcriptional activation (Sidorova and Breeden, 2002). However, the dearth of physical interactions between genetically identified regulators has been an impediment to building a detailed model of Start. Whi5 provides this missing physical connection. Because the ANK repeats of Swi6 antagonize the intrinsic transcriptional activation function of Swi6 (Sedgwick et al., 1998), Whi5 may interact

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(F) Phosphorylation of SBF is sufficient to cause Whi5 dissociation. The same Cln2-Cdc28 reactions were carried out as in (C), except that Whi5[12AGST] was bound to Swi4[FLAG]-Swi6 complex on anti-FLAG resin.
(G) Elimination of consensus CDK phosphorylation sites in both Whi5 and Swi6 causes a severe growth defect. An isogenic wild-type strain (BY4742) and a swi6Δ <swi6[SA4]> (MTY2574, pBD1756) bearing either *GAL1-WHI5* (pMT3445) or *GAL1-WHI5[12A]* (pMT3455) were spotted in serial 10-fold dilutions on either glucose or galactose medium and incubated at 30°C for 48 hr.

with this region, which probably functions as a platform for G1/S regulators, including Stb1 and the general transcriptional machinery (Macpherson et al., 2000; Sanders et al., 2002). As loss of *WHI5* is not entirely epistatic to overproduction of either *CLN3-1* or *BCK2*, we infer the existence of additional CDK-dependent activation steps such as phosphorylation of SBF/MBF or elimination of other minor SBF/MBF inhibitors. Whi5 has limited similarity to another budding yeast protein called Srl3, originally isolated as a multicopy suppressor of *rad53Δ* lethality and since identified in a complex with the Cdc28 regulatory subunit Cks1 (Gavin et al., 2002). However, the *whi5Δ srl3Δ* double mutant has no growth defect and is no smaller than the *whi5* single mutant (J.L.N. and M.T., unpublished data). With Whi5 in hand, the G1/S regulatory apparatus may be more amenable to deconstruction, including the still-mysterious mechanism that determines the timing of Cln3-Cdc28 activation in late G1 phase.

Onset of G1/S Transcription

As perhaps befits a linchpin in the cell cycle commitment decision, Whi5 is regulated by at least two CDK-dependent mechanisms. Disengagement of Whi5 from SBF appears to proceed via multiple redundant phosphorylation events, since elimination of all sites on Whi5 or multiple sites on Swi4 and Swi6 (Wijnen et al., 2002) is without any overt phenotypic consequence. Indeed, dissociation of the Whi5-SBF complex in vitro can be triggered in the absence of Whi5 phosphorylation, and the combination of Whi5 and Swi6 phosphorylation site mutants is nearly lethal. Given the multiple phosphorylation events needed for activation of G1/S transcription and provided that CDK-dependent phosphorylation is distributive, the overall forward reaction is predicted to exhibit ultrasensitivity with respect to kinase concentration, thereby rendering the onset of transcription more switch-like (Ferrell, 1996). An analogous multisite phosphorylation mechanism appears to operate for the recognition of Sic1 by the SCF^Cdc4 ubiquitin ligase (Verma et al., 1997; Nash et al., 2001a). By further analogy to Sic1, we initially anticipated that regulation of Whi5 might depend on SCF^Cdc4-mediated ubiquitination of phospho-Whi5. Although we have found that Whi5 is bound and ubiquitinated in a phosphorylation-dependent manner by SCF^Cdc4 in vitro, Whi5 abundance is not increased in a *cdc4* arrest, nor does Whi5 appear to be unstable in vivo (X.T. and M.T., unpublished data). Whether or not SCF^Cdc4-dependent ubiquitination of a small pool of Whi5 refines the onset of G1/S transcription remains to be determined.

CDK-dependent nuclear export provides a second layer of control over Whi5. The timing of Whi5 transit into and out of the nucleus derives from the fact that Whi5 is excluded from the nucleus by all forms of cyclin-Cdc28 activity. At the end of mitosis, once CDK activity is quelled, a preexisting cytoplasmic pool of Whi5 immediately enters the nucleus to ensure that transcription is not activated as soon as SBF and MBF engage promoter DNA in early G1 phase. In contrast, Sic1 accumulation in early G1 phase lags slightly behind Whi5 because it requires both Swi5-dependent transcription and protein synthesis (Visintin et al., 1998). Conversely, at the end

of G1 phase, Whi5 exits the nucleus well before bud emergence, whereas Sic1 is eliminated just prior to bud emergence. This sequence reflects the fact that Whi5 dissociation from SBF/MBF is triggered immediately upon activation of Cln3-Cdc28, while Sic1 elimination via SCF^Cdc4 is catalyzed primarily by Cln1/2-Cdc28, which in turn requires SBF-dependent transcription of *CLN1/2*. Aside from placing a clamp on SBF/MBF activation in early G1, the regulated translocation of Whi5 to and from the cytoplasmic compartment may also engender switch-like behavior (Ferrell, 1998). Regardless, the sundering of the Whi5-SBF complex resides close to if not at the very apex of the cell cycle commitment decision.

The CDK dependence of Whi5 import and/or export parallels that of Swi5 (Moll et al., 1991); however, the presumptive karyopherins that mediate phosphorylation-dependent redistribution of Swi5 are unknown. Intriguingly, deletion of *KAP122*, which encodes an uncharacterized karyopherin, causes a Whi phenotype (Jorgensen et al., 2002) and was a weak hit in the SCB reactivation screen (Supplemental Table S1). Swi6 is also excluded from the nucleus after G1 phase in a phosphorylation-dependent manner (Sidorova et al., 1995), probably via the nuclear export factor Msn5 (Queralt and Igual, 2003). It is very likely that Whi5 is driven back into the nucleus by the Cdc14 phosphatase, which is activated upon mitotic exit. Cdc14 is required for nuclear reentry of Swi5 and Cdc6 at the end of mitosis, and Cdc14 preferentially dephosphorylates Sic1, Swi5, and Swi6 in vitro (Visintin et al., 1998; Geymonat et al., 2004). Consistently, overexpression of *CDC14* represses G1/S transcripts in cells arrested by a *cdc4-1* mutation (P. Jorgensen and M.T., unpublished data). The dual control of Whi5 by CDK activity is reminiscent of the transcription factor Pho4, which is subject to phosphorylation-dependent regulation by the CDK enzyme Pho85 at both the level of nuclear export/import and protein interactions at the promoter (Komeili and O'Shea, 1999). Similarly, the apparently pleonastic phosphorylation of Whi5 and SBF/MBF is analogous to the CDK-dependent inhibition of DNA replication, which is enforced via redundant phosphorylation events on multiple replication factors (Nguyen et al., 2001).

Analogies between Whi5 and Rb Family Proteins

The Whi5 pathway in budding yeast is organized in a strikingly similar manner to the Rb pathway in metazoans (Figure 7B). Dissociation of the Rb-E2F interaction requires CDK-dependent phosphorylation on multiple sites (Brown et al., 1999), as the case appears for the Whi5-SBF interaction. In both yeast and metazoans, a potential positive feedback loop of G1/S cyclin activity may sharpen the onset, as opposed to the timing, of G1/S transcription (Tyers et al., 1993; Dirick et al., 1995; Stuart and Wittenberg, 1995; Kaelin, 1999). Like Rb family proteins, Whi5 is rendered inactive in the face of all forms of CDK activity, from the end of G1 phase until the end of mitosis. Intriguingly, Cln3 and Cln1/2 mediate stepwise phosphorylation of mammalian Rb expressed in yeast, not unlike its bona fide regulation by cyclin D and cyclin E (Hatakeyama et al., 1994). Moreover, the triple Rb family member knockout in mice causes a pronounced small cell size phenotype (Dannenberg et

al., 2000; Sage et al., 2000), as do mutations in *mat3*, the *Chlamydomonas* Rb homolog (Umen and Goodenough, 2001). The pheromone resistance of a *whi5Δ* strain is also analogous to the requirement for Rb in G1 arrest of mammalian cells (Sage et al., 2000). Rb function has been elaborated to many other facets, including roles in differentiation, growth inhibition, apoptosis, and the DNA damage response (Stevaux and Dyson, 2002). Such potential roles for Whi5 remain to be explored.

Conservation of Regulatory Architecture

Despite the functional concordance between Rb and Whi5, there is no obvious sequence conservation between the two proteins. Similarly, neither yeast CDK inhibitors nor SBF/MBF bears any resemblance to their metazoan counterparts. The absence of overt orthology, if anything, underscores the conserved genetic logic of the Whi5 and Rb pathways in G1 phase control. Given the critical roles played by Whi5 and Rb in cell cycle regulation, it is also striking that deletion of either gene has, at best, a marginal effect on cell viability (Kaelin, 1999). This feature is shared by a number of mammalian cell cycle regulatory factors, whose loss has phenotypic consequences primarily in the context of cancer, including *p53*, *Arf*, *E2f-1*, *Cyclin D*, *Cyclin E*, and *Cdk2* (Sherr and McCormick, 2002; Hinds, 2003). Presumably this dispensability derives from the accretion of plastic regulatory layers on the essential core machinery that carries out cell division (Kirschner and Gerhart, 1998). Given these evolutionary considerations, further investigation of the nature of cell cycle commitment in yeast may unveil additional regulatory principles that pertain to metazoan cell division (Pardee, 1989).

Experimental Procedures

Yeast Culture, Strains, and Plasmids

Growth medium, FACS analysis of DNA content, and cell size measurements were described previously (Jorgensen et al., 2002). Yeast integrations were confirmed by flanking PCR, and mutagenized DNA fragments were sequenced in their entirety. Strains and plasmids used in this study are listed in Supplemental Tables S2 and S3. Details of construction will be provided upon request. A modified synthetic genetic array (SGA) method was used to identify deletion strains in which the transcriptional defect resulting from *cln3Δ* is suppressed (Tong et al., 2001). A strain harboring an integrated copy of the *HIS3* reporter gene under the control of four consensus SCB elements (BY2054) was mated to an array of 4812 yeast deletion strains (Giaever et al., 2002). Diploids were selected on medium containing G418 (200 mg/L, Invitrogen) and clonNAT (100 mg/L, Werner BioAgents). Double mutant *MATa SCB:HIS3::URA3 cln3Δnat xxxΔkan* haploids were isolated through successive pinning steps on selective media, then pinned onto medium lacking histidine and containing 30 mM 3′-aminotriazole (3′-AT). β-galactosidase assays were performed as described (Costanzo et al., 2003). Standard methods were used for fluorescence and differential interference microscopy (Nash et al., 2001a). For time course studies, individual cells were embedded in 0.4% agar under a cover slip and fluorescent images acquired over three vertical sections. For dual Sic1-CFP Whi5-YFP images, images over five Z planes (0.4 μm apart) were taken in the following order: YFP (excitation 500 nm/emission 535 nm, exposure time 125 ms); black YFP reference (no excitation/535 nm, 125 ms); YFP autofluorescence reference (350/535 nm, 400 ms); CFP (430/470 nm, 125 ms); black CFP reference (no excitation/470 nm, 125 ms); and CFP autofluorescence reference (350/470 nm; 1000 ms). Images were corrected for shading and autofluorescence, then Z sections with maximum average signal in the nuclear region

used to calculate intensity ratios. Only cells in which either nuclear signal was discernible were analyzed.

Cell Synchronization, RNA Analysis, and Protein Analysis

Cultures were synchronized by centrifugal elutriation or α factor arrest (Tyers et al., 1993). Wild-type and *whi5Δ* cultures used for elutriation were grown to near saturation overnight in order to enrich for small G1 phase cells. Northern blots of total RNA were probed for *ACT1*, *CLN2*, and *RNR1* and quantitated on a phosphorimager (Tyers et al., 1993). Immunoaffinity purification of FLAG-tagged protein complexes and identification of associated species by mass spectrometry was performed as described (Ho et al., 2002). Proteins were detected with 9E10 anti-Myc, 12CA5 anti-HA, and M2 anti-FLAG monoclonal antibodies or affinity-purified polyclonal antibodies (Ho et al., 1999, 2002). Recombinant proteins were produced in a Codon+ BL21 bacterial strain or in insect cells infected with baculovirus expression vectors (Nash et al., 2001a). Kinase assays and protein binding reactions were carried out in standard buffers (Tyers et al., 1992; Nash et al., 2001a).

Promoter Binding and Chromatin Immunoprecipitation

Conventional chromatin immunoprecipitation and PCR of sequences from pLGΔSS were performed as described (Costanzo et al., 2003). Quantitative real-time PCR was carried out by dual fluorogenic reporter TaqMan assay in an ABI PRISM 7900HT Sequence Detection System as recommended by Perkin-Elmer/Applied Biosystems. Target gene probes were labeled with 6-carboxyfluorescin (FAM) and black hole quencher (BHQ), and internal control probe for a transcriptionally inert region of chromosome 2 was labeled with TET (Texas Red-derived fluorescent dye) and BHQ. Probes and flanking primers were designed in the −500 to 0 bp promoter region of the test genes using Primer Express Software (Perkin Elmer) and are available on request. Capture efficiency was calculated as the ratio of immunoprecipitated versus total DNA for probe signal subtracted by that for a transcriptionally inert region of chromosome II signal at the midpoint of each reaction curve. For in vitro DNA capture assays, plasmids were restriction digested, labeled with [^{32}P]-α-dATP by Klenow fragment, incubated with purified recombinant proteins, and captured on either anti-FLAG or glutathione resin (Johnson and Herskowitz, 1985). After binding, beads were washed three times; then resuspended in 10 mM Tris-Cl (pH 7.4), 20 mM NaCl, 0.1% SDS; heated to 65°C for 10 min; and extracted with phenol/chloroform. DNA was precipitated, resolved on a 4% native polyacrylamide gel, and visualized by autoradiography.

References

Amon, A., Tyers, M., Futcher, B., and Nasmyth, K. (1993). Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell *74*, 993–1007.

Baetz, K., Moffat, J., Haynes, J., Chang, M., and Andrews, B. (2001). Transcriptional coregulation by the cell integrity mitogen-activated

protein kinase Slt2 and the cell cycle regulator Swi4. Mol. Cell. Biol. *21*, 6515–6528.

Breeden, L. (1996). Start-specific transcription in yeast. Curr. Top. Microbiol. Immunol. *208*, 95–127.

Brown, V.D., Phillips, R.A., and Gallie, B.L. (1999). Cumulative effect of phosphorylation of pRB on regulation of E2F activity. Mol. Cell. Biol. *19*, 3246–3256.

Cosma, M.P., Tanaka, T., and Nasmyth, K. (1999). Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell *97*, 299–311.

Cosma, M.P., Panizza, S., and Nasmyth, K. (2001). Cdk1 triggers association of RNA polymerase to cell cycle promoters only after recruitment of the mediator by SBF. Mol. Cell *7*, 1213–1220.

Costanzo, M., Schub, O., and Andrews, B. (2003). G1 transcription factors are differentially regulated in *S. cerevisiae* by the Swi6-binding protein Stb1. Mol. Cell. Biol. *23*, 5064–5077.

Cross, F.R. (1995). Starting the cell cycle: what's the point? Curr. Opin. Cell Biol. *7*, 790–797.

Dannenberg, J.H., van Rossum, A., Schuijff, L., and te Riele, H. (2000). Ablation of the retinoblastoma gene family deregulates G(1) control causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev. *14*, 3051–3064.

Dietrich, F.S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pohlmann, R., Luedi, P., Choi, S., et al. (2004). The Ashbya gossypii genome as a tool for mapping the ancient Saccharomyces cerevisiae genome. Science *304*, 304–307.

Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of the Cln-Cdc28 kinases at the start of the cell cycle of *Saccharomyces cerevisiae*. EMBO J. *14*, 4803–4813.

Ferrell, J.E., Jr. (1996). Tripping the switch fantastic: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem. Sci. *21*, 460–466.

Ferrell, J.E., Jr. (1998). How regulated protein translocation can produce switch-like responses. Trends Biochem. Sci. *23*, 461–465.

Gavin, A.C., Bosche, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., Schultz, J., Rick, J.M., Michon, A.M., Cruciat, C.M., et al. (2002). Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature *415*, 141–147.

Geymonat, M., Spanos, A., Wells, G.P., Smerdon, S.J., and Sedgwick, S.G. (2004). Clb6/Cdc28 and Cdc14 regulate phosphorylation status and cellular localization of Swi6. Mol. Cell. Biol. *24*, 2277–2285.

Giaever, G., Chu, A.M., Ni, L., Connelly, C., Riles, L., Veronneau, S., Dow, S., Lucau-Danila, A., Anderson, K., Andre, B., et al. (2002). Functional profiling of the Saccharomyces cerevisiae genome. Nature *418*, 387–391.

Harrington, L.A., and Andrews, B.J. (1996). Binding to the yeast Swi4,6-dependent cell cycle box, CACGAAA, is cell cycle regulated *in vivo*. Nucleic Acids Res. *24*, 558–565.

Hatakeyama, M., Brill, J.A., Fink, G.R., and Weinberg, R.A. (1994). Collaboration of G1 cyclins in the functional inactivation of the retinoblastoma protein. Genes Dev. *8*, 1759–1771.

Hinds, P.W. (2003). Cdk2 dethroned as master of S phase entry. Cancer Cell *3*, 305–307.

Ho, Y., Costanzo, M., Moore, L., Kobayashi, R., and Andrews, B. (1999). Regulation of transcription at the *Saccharomyces cerevisiae* Start transition by Stb1, a Swi6-binding protein. Mol. Cell. Biol. *19*, 5267–5278.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutillier, K., et al. (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectometry. Nature *415*, 180–183.

Jeoung, D., Oehlen, L.J., and Cross, F.R. (1998). Cln3-associated kinase activity in Saccharomyces cerevisiae is regulated by the mating factor pathway. Mol. Cell. Biol. *18*, 433–441.

Johnson, A.D., and Herskowitz, I. (1985). A repressor (MAT alpha2 Product) and its operator control expression of a set of cell type specific genes in yeast. Cell *42*, 237–247.

Johnston, G.C., Ehrhardt, C.W., Lorinez, A., and Carter, B.L.A. (1979). Regulation of cell size in the yeast Saccharomyces cerevisiae. J. Bacteriol. *137*, 1–5.

Jorgensen, P., Nishikawa, J.L., Breitkreutz, B., and Tyers, M. (2002). Systematic identification of pathways that couple cell growth and division in yeast. Science *297*, 395–400.

Kaelin, W.G., Jr. (1999). Functions of the retinoblastoma protein. Bioessays *21*, 950–958.

Kirschner, M., and Gerhart, J. (1998). Evolvability. Proc. Natl. Acad. Sci. USA *95*, 8420–8427.

Koch, C., Schleiffer, A., Ammerer, G., and Nasmyth, K. (1996). Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G2. Genes Dev. *10*, 129–141.

Komeili, A., and O'Shea, E. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor, Pho4. Science *284*, 977–980.

Macpherson, N., Measday, V., Moore, L., and Andrews, B. (2000). A *taf17* mutant requires the Swi6 transcriptional activator for viability and shows defects in cell cycle regulated transcription. Genetics *154*, 1561–1576.

Makhnevych, T., Lusk, C.P., Anderson, A.M., Aitchison, J.D., and Wozniak, R.W. (2003). Cell cycle regulated transport controlled by alterations in the nuclear pore complex. Cell *115*, 813–823.

McInerny, C.J., Partridge, J.F., Mikesell, G.E., Creemer, D.P., and Breeden, L.L. (1997). A novel Mcm1-dependent promoter element in the *SWI4, CLN3, CDC6*, and *CDC47* promoters activates M/G1-specific transcription. Genes Dev. *11*, 1277–1288.

Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. Cell *66*, 743–758.

Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, B. (1988). The *WHI1+* gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. *7*, 4335–4346.

Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001a). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.

Nash, R.S., Volpe, T., and Futcher, B. (2001b). Isolation and characterization of *WHI3*, a size-control gene of *Saccharomyces cerevisiae*. Genetics *157*, 1469–1480.

Nasmyth, K. (1996). At the heart of the budding yeast cell cycle. Trends Genet. *12*, 405–412.

Neufeld, T.P., de la Cruz, A.F., Johnston, L.A., and Edgar, B.A. (1998). Coordination of growth and cell division in the *Drosophila* wing. Cell *93*, 1183–1193.

Nguyen, V.Q., Co, C., and Li, J.J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068–1073.

Pardee, A. (1989). G1 events and regulation of cell proliferation. Science *246*, 603–608.

Queralt, E., and Igual, J.C. (2003). Cell cycle activation of the Swi6 transcription factor is linked to nucleoplasmic shuttling. Mol. Cell. Biol. *23*, 3126–3140.

Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R.A., and Dynlacht, B.D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. Genes Dev. *16*, 245–256.

Sage, J., Mulligan, G.J., Attardi, L.D., Miller, A., Chen, S., Williams, B., Theodorou, E., and Jacks, T. (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. Genes Dev. *14*, 3037–3050.

Sanders, S.L., Jennings, J., Canutescu, A., Link, A.J., and Weil, P.A. (2002). Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectometry. Mol. Cell. Biol. *22*, 4723–4738.

Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40^{SIC1} controls the G1 to S transition in *S. cerevisiae*. Cell *79*, 233–244.

Sedgwick, S.G., Taylor, I.A., Adam, A.C., Spanos, A., Howell, S., Morgan, B.A., Treiber, M.K., Kanuga, N., Banks, G.R., Foord, R., and Smerdon, S.J. (1998). Structural and functional architecture of the yeast cell-cycle transcription factor Swi6. J. Mol. Biol. *281*, 763–775.

Sherr, C.J., and McCormick, F. (2002). The RB and p53 pathways in cancer. Cancer Cell *2*, 103–112.

Sidorova, J.M., and Breeden, L.L. (1997). Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. Genes Dev. *11*, 3032–3045.

Sidorova, J.M., and Breeden, L.L. (2002). Precocious S-phase entry in budding yeast prolongs replicative state and increases dependence upon Rad53 for viability. Genetics *160*, 123–136.

Sidorova, J.M., Mikesell, G.E., and Breeden, L. (1995). Cell cycle-regulated phosphorylation of Swi6 controls its nuclear localization. Mol. Biol. Cell *6*, 1641–1658.

Spellman, P.T., Sherlock, G., Zhang, M.Q., Vishwanath, R.I., Anders, K., Eisen, M.B., Brown, P.O., Botstein, D., and Futcher, B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. Mol. Biol. Cell *9*, 3273–3297.

Stevaux, O., and Dyson, N.J. (2002). A revised picture of the E2F transcriptional network and RB function. Curr. Opin. Cell Biol. *14*, 684–691.

Stuart, D., and Wittenberg, C. (1995). *CLN3*, not positive feedback, determines the timing of *CLN2* transcription in cycling cells. Genes Dev. *9*, 2780–2794.

Tong, A.H., Evangelista, M., Parsons, A.B., Xu, H., Bader, G.D., Page, N., Robinson, M., Raghibizadeh, S., Hogue, C.W., Bussey, H., et al. (2001). Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science *294*, 2364–2368.

Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nat. Rev. Mol. Cell Biol. *3*, 11–20.

Tyers, M., Tokiwa, G., Nash, R., and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. *11*, 1773–1784.

Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. *12*, 1955–1968.

Umen, J.G., and Goodenough, U.W. (2001). Control of cell division by a retinoblastoma protein homolog in Chlamydomonas. Genes Dev. *15*, 1652–1661.

Verma, R., Annan, R.S., Huddleston, M.J., Carr, S.A., Reynard, G., and Deshaies, R.J. (1997). Phosphorylation of Sic1 by G1 Cdk required for its degradation and entry into S phase. Science *278*, 455–460.

Visintin, R., Craig, K., Hwang, E.S., Prinz, S., Tyers, M., and Amon, A. (1998). The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol. Cell *2*, 709–718.

Wijnen, H., and Futcher, B. (1999). Genetic analysis of the shared role of *CLN3* and *BCK2* at the G1-S transition in *Saccharomyces cerevisiae*. Genetics *153*, 1131–1143.

Wijnen, H., Landman, A., and Futcher, B. (2002). The G1 cyclin Cln3 promotes cell cycle entry via the transcription factor Swi6. Mol. Cell. Biol. *22*, 4402–4418.

Zhang, J., Schneider, C., Ottmers, L., Rodriguez, R., Day, A., Markwardt, J., and Schneider, B.L. (2002). Genomic scale mutant hunt identifies cell size homeostasis genes in *S. cerevisiae*. Curr. Biol. *12*, 1992–2001.