Cell, Vol. 117, 887–898, June 25, 2004, Copyright ©2004 by Cell Press

Cln3 Activates G1-Specific Transcription via Phosphorylation of the SBF Bound Repressor Whi5

Robertus A.M. de Bruin,[1] W. Hayes McDonald,[2,3] Tatyana I. Kalashnikova,[1] John Yates III,[2] and Curt Wittenberg[1,2,*]

[1]Department of Molecular Biology
[2]Department of Cell Biology
The Scripps Research Institute
10550 North Torrey Pines Road
La Jolla, California 92037

Summary

G1-specific transcriptional activation by Cln3/CDK initiates the budding yeast cell cycle. To identify targets of Cln3/CDK, we analyzed the SBF and MBF transcription factor complexes by multidimensional protein interaction technology (MudPIT). Whi5 was identified as a stably bound component of SBF but not MBF. Inactivation of Whi5 leads to premature expression of G1-specific genes and budding, whereas overexpression retards those processes. Whi5 inactivation bypasses the requirement for Cln3 both for transcriptional activation and cell cycle initiation. Whi5 associates with G1-specific promoters via SBF during early G1 phase, then dissociates coincident with transcriptional activation. Dissociation of Whi5 is promoted by Cln3 in vivo. Cln/CDK phosphorylation of Whi5 in vitro promotes its dissociation from SBF complexes. Mutation of putative CDK phosphorylation sites, at least five of which are phosphorylated in vivo, strongly reduces SBF-dependent transcription and delays cell cycle initiation. Like mammalian Rb, Whi5 is a G1-specific transcriptional repressor antagonized by CDK.

Introduction

In budding yeast, as in most cells of metazoan organisms, cell proliferation is regulated during late G1 phase in response to a variety of environmental and internal conditions (reviewed by Wittenberg and Flick [2003]). Preconditions must be met prior to initiation of a new cell cycle. In budding yeast, failure to satisfy those criteria can lead to aberrant or incomplete cell cycle events (Mendenhall and Hodge, 1998). In metazoans, the loss of control over cell cycle initiation can, in addition, lead to errors in development, proliferative diseases, and ultimately death (reviewed by Ho and Dowdy [2002]).

The temporally controlled accumulation of families of genes encoding components of the cellular machinery required for subsequent events ensures that their functions are implemented during the appropriate interval. In fact, the transcriptional activation of G1-specific genes is the earliest indicator of cell cycle initiation, often referred to as Start (reviewed by Breeden [2003] and Wittenberg and Flick [2003]). More than 200 genes fall into the G1-specific gene family (Cho et al., 1998; Spellman et al., 1998). Together, they govern the events associated with cell cycle initiation, including DNA replication, bud morphogenesis, and duplication of spindle pole bodies and thereby impact most subsequent events of the cell cycle.

Expression of G1-specific genes depends upon the heterodimeric transcription factors SBF and MBF, each comprised of Swi6 and a sequence-specific DNA binding protein, Swi4 or Mbp1, respectively (Breeden, 2003). Binding of those transcription factors to one or more cognate sequences in the promoters of G1-specific genes is essential for the proper temporal pattern of transcription. Consequently, inactivation of Mbp1 and Swi4 leads to loss of viability and arrest during G1 phase (Koch et al., 1993). However, neither Swi4 nor Mbp1 alone is essential. Nevertheless, there are distinct differences in the patterns of gene expression in the individual mutants (Costanzo et al., 2003; Koch et al., 1993), consistent with the distribution of MBF and SBF binding sites among G1-specific genes (Iyer et al., 2001; Simon et al., 2001; Spellman et al., 1998). Finally, SBF and MBF appear to be subject to differential regulation (Amon et al., 1993; Costanzo et al., 2003). Nevertheless, the two factors do show limited functional overlap under some conditions (Flick et al., 1998; Partridge et al., 1997).

Despite their well-established roles in G1-specific transcription, binding of SBF and MBF is insufficient for transcriptional activation (Cosma et al., 2001; Harrington and Andrews, 1996; Koch et al., 1993). Instead, efficient and timely transcriptional activation requires the G1 cyclin Cln3 (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993) and its cognate cyclin-dependent protein kinase (CDK), Cdc28 (Koch et al., 1996; Marini and Reed, 1992). Activation of the Cln3/CDK promotes the formation of the RNA polymerase II holoenzyme at the basal promoters of SBF and MBF bound genes and thereby transcriptional activation (Cosma et al., 2001).

Consistent with its role in G1-specific transcription, Cln3 is a dose-dependent activator of cell cycle initiation (Cross, 1988; Nash et al., 1988; Tyers et al., 1993). Inactivation of *CLN3* causes a dramatic cell size increase, whereas additional copies of *CLN3* promote budding at progressively smaller cell size. Those effects result largely from its effect on G1-specific transcription (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993) exerted via SBF and MBF (Wijnen et al., 2002). In the most extreme case, hyperstabilization of Cln3 leads not only to an dramatic decrease in the cell size required for budding and entry into S phase but also to resistance to mating pheromone-induced G1 arrest (Cross, 1988; Nash et al., 1988). Although *CLN3* is expressed throughout the cell cycle, expression is maximal as cells enter G1 phase (MacKay et al., 2001; McInerny et al., 1997). Cln3 protein levels are low and highly sensitive to changes in the nutritional and protein-synthetic capacity to cells (Gallego et al., 1997; Polymenis and Schmidt, 1997). Because of the tight coupling between Cln3 abundance and its synthesis rate and because transcriptional

*Correspondence: curtw@scripps.edu
[3]Present address: Chemical Sciences Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, Tennessee 37831.

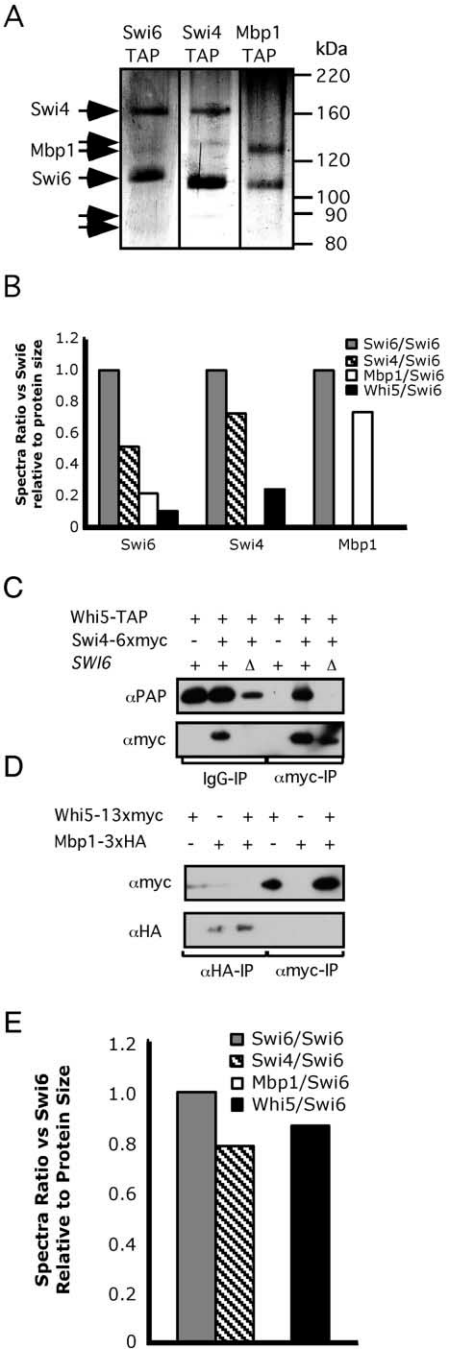

Figure 1. Whi5 Is a Component of SBF

(A) TAP of SBF and/or MBF components (arrows: Swi6, Swi4 and/or Mbp1) plus additional unknown proteins (arrows) as detected by silver staining of TAP SBF/MBF purified samples separated by SDS-PAGE.

(B) Whi5 associates specifically with Swi6 and Swi4. Bars represent number of spectra of proteolytic peptides having a mass consistent with peptides predicted for Swi6, Swi4, Mbp1, or Whi5 as identified by MudPIT analysis. All samples were normalized to the common component, Swi6, taking into consideration both number of spectra and protein size.

(C) Whi5 interacts with SBF. Extracts were prepared from wild-type or *swi6*Δ strains carrying *WHI5*-TAP together with *SWI4*-6xmyc. Anti-TAP (IgG-IP) and anti-myc (αmyc-IP) immune complexes from asynchronous cultures were probed with αPAP to detect Whi5-TAP or with anti-myc to detect Swi4-6xmyc.

(D) Whi5 does not interact with Mbp1. Extracts were prepared from wild-type strains carrying *MBP1*-3xHA, *WHI5*-13xmyc, or both. Anti-HA (αHA-IP) and anti-myc (αmyc-IP) immune complexes from asynchronous cultures were probed with anti-myc to detect Whi5-13xmyc or with anti-HA to detect Mbp1-3xHA.

(E) Whi5 associates specifically with Swi6 and Swi4. The number of spectra representing known SBF and MBF components obtained from the MudPIT analysis of Whi5-TAP was normalized to Swi6 (based upon both spectra number and protein size).

activation is highly sensitive to Cln3 level, it is well suited as a sensor of cell growth.

Despite the requirement for Cln3 for timely activation of G1-specific genes, it is not absolutely required for expression of those genes or for cell viability. However, it becomes essential in the absence of *CLN1* and *CLN2* (Richardson et al., 1989), G1-specific genes encoding G1 cyclins, or *BCK2* (Di Como et al., 1995; Epstein and Cross, 1994). *BCK2* also becomes essential in cells lacking *SWI4* or *SWI6* (Wijnen and Futcher, 1999). *CLN1* and *CLN2* are sufficient for transcriptional activation when *CLN3* is inactive (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993). The mechanism by which Bck2 promotes transcription and cell cycle initiation is unclear, but it appears to be at least partially independent of SBF and MBF. Despite its apparent redundancy with Cln3 and Swi6, Bck2 plays a relatively modest role in their presence.

The mechanism of transcriptional activation via Cln3/CDK has yet to be established. Although both Swi4 and Swi6 are phosphorylated in a cell cycle-dependent manner at CDK consensus phosphorylation sites, those sites appear not to be required for transcriptional activation (Koch et al., 1996; Sidorova et al., 1995; Wijnen et al., 2002). Furthermore, Cln3/CDK appears not to bind directly to SBF or MBF or to promoters of target genes (Wijnen et al., 2002; K. Flick and C.W., unpublished data). This has led to the suggestion that one or more regulatory factors intervene between the G1 cyclin/CDK and the G1-specific transcription factors. A similar system has been well established in metazoans, in which the tumor suppressor Rb represses E2F-dependent transcription during G1 phase but is then inactivated via phosphorylation by cyclin D/Cdk4, leading to the activation of genes required for entry into S phase (reviewed by Dyson [1998] and Nevins [2001]). In fact, that mammalian regulatory cascade has been reconstructed at least in part in yeast cells (Hatakeyama et al., 1994).

In the interest of identifying putative targets of the Cln3/CDK, we have isolated and characterized components of the SBF transcription factor complex. Among the proteins forming a complex with SBF was Whi5, identified in genomic screens for mutants that result in an abnormally small cell size (Jorgensen et al., 2002; Zhang et al., 2002). We show here that Whi5 associates with the promoter bound SBF transcription factor to repress transcription. Furthermore, Cln3 antagonizes binding of Whi5 to SBF-responsive promoters and its activity as a transcriptional repressor. We suggest that Whi5, like Rb, represses G1-specific transcription during early G1 phase and thereby restrains cell cycle initiation and cell proliferation until it is inactivated by G1 cyclin-dependent protein kinase.

Results

Identification of Components of SBF and MBF

To identify potential targets of Cln3/CDK required for activation of G1-specific transcription, we sought to define components of the SBF and MBF transcription factors from cells that were either arrested in G1 phase prior to transcriptional activation or released into late G1 phase, during which G1-specific transcription is maximally activated. To achieve this, Swi4, Swi6, and Mbp1, the known components of the G1-specific transcription factors, were tagged at the carboxyterminus with the tandem affinity purification (TAP) epitope at their genomic loci in a *cln1*Δ *cln2*Δ strain carrying *CLN3* under control of the *GAL1* promoter as its only source of G1 cyclin. Cells expressing each of the epitope-tagged transcription factor subunits were either arrested during G1 phase by depletion of Cln3 on raffinose or released from G1 phase arrest for 20 min (the predetermined interval required for maximal transcriptional activation) by addition of galactose and protein complexes purified by TAP (Rigaut et al., 1999). Silver-stained SDS-PAGE of the TAP-purified fractions shows that each contains, in addition to the epitope-tagged component, the expected SBF or MBF components plus additional unknown copurified proteins (Figure 1A).

Identification of the components of the transcription factor complexes was accomplished using the mass spectrometry-based multidimensional protein interaction technology (MudPIT; Wolters et al., 2001), resulting in the identification of each of the expected SBF and MBF components (Swi6, Swi4, and/or Mbp1; Figure 1B) along with several components that, to our knowledge, are novel. Those components were classified as SBF specific (Swi4 and Swi6 fractions), MBF specific (Mbp1 and Swi6 fractions), Swi4 specific, or Mbp1 specific. No Swi6-specific interactors were identified. Unexpectedly, all but one of those interactors detected in this screen occur with comparable confidence in cells having active or inactive SBF/MBF (data not shown).

Two previously studied proteins, Stb1 and Whi5, were shown to interact predominantly with MBF or specifically with SBF, respectively. Stb1 has been reported to be an MBF-specific transcriptional activator (Costanzo et al., 2003). Whi5 was identified with the highest confidence of any component in SBF complexes other than the known subunits Swi4 and Swi6 (Figure 1B), based upon both peptide sequence coverage and spectral quality. In contrast, Whi5 was not identified in any of the Mbp1-containing complexes analyzed. Surprisingly, despite prior analysis of SBF complexes, an interaction with Whi5 had not been reported (Gavin and Superti-Furga, 2003; Ho et al., 2002).

Whi5 Is a Component of SBF

To confirm the specific interaction of Whi5 with SBF detected by MudPIT, we evaluated the interaction of those proteins by coimmunoprecipitation (coIP). Immune complexes containing TAP-tagged Whi5 also contained Myc-tagged Swi6 (data not shown) and Myc-tagged Swi4 (Figure 1C), whereas no Mbp1-HA was detected (Figure 1D). Accordingly, Whi5-TAP coimmunoprecipitated with Swi6-6xmyc (data not shown) and Swi4-6xmyc (Figure 1C) but not with Mbp1-3xHA (Figure 1D). To determine whether Whi5 interacts with the intact SBF complex or with Swi6 or Swi4 independently, we repeated the immunoprecipitation in strains carrying a deletion mutant of the untagged SBF component. We observed that inactivation of either *SWI6* or *SWI4* abolishes the interaction between Whi5 and the remaining SBF component (Figure 1C and data not shown). Finally, analysis of Whi5-TAP complexes by MudPIT resulted in the identification of both Swi6 and Swi4 with high confidence, whereas Mbp1 was not detected (Figure 1E). Together, these data establish that Whi5 is a component of SBF.

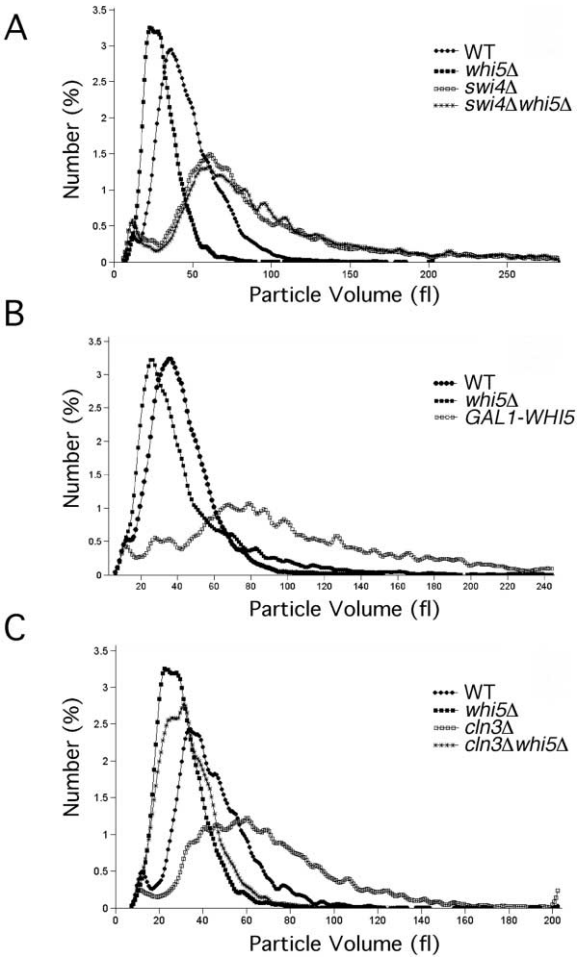

Figure 2. Cell Size Analysis of Whi5 Mutants

The cell volume distribution (fl) of log phase cultures of each of the indicated strains grown in YEPD (except as indicated) was determined using a Coulter Z2 Particle Analyzer. (A) Small cell size of *whi5*Δ depends upon SBF. (B) Overexpression of *WHI5* increases cell size. Cells were grown in galactose to induce *GAL-WHI5*. (C) The size increase resulting from *cln3*Δ is suppressed by *whi5*Δ.

Whi5 Inhibits SBF-Dependent G1-Specific Transcription

WHI5 (*YOR083w*) was identified previously based upon the small cell size caused by a null mutation (Jorgensen et al., 2002). Further analysis suggested that Whi5 might act as an inhibitor of G1-specific transcription. We find

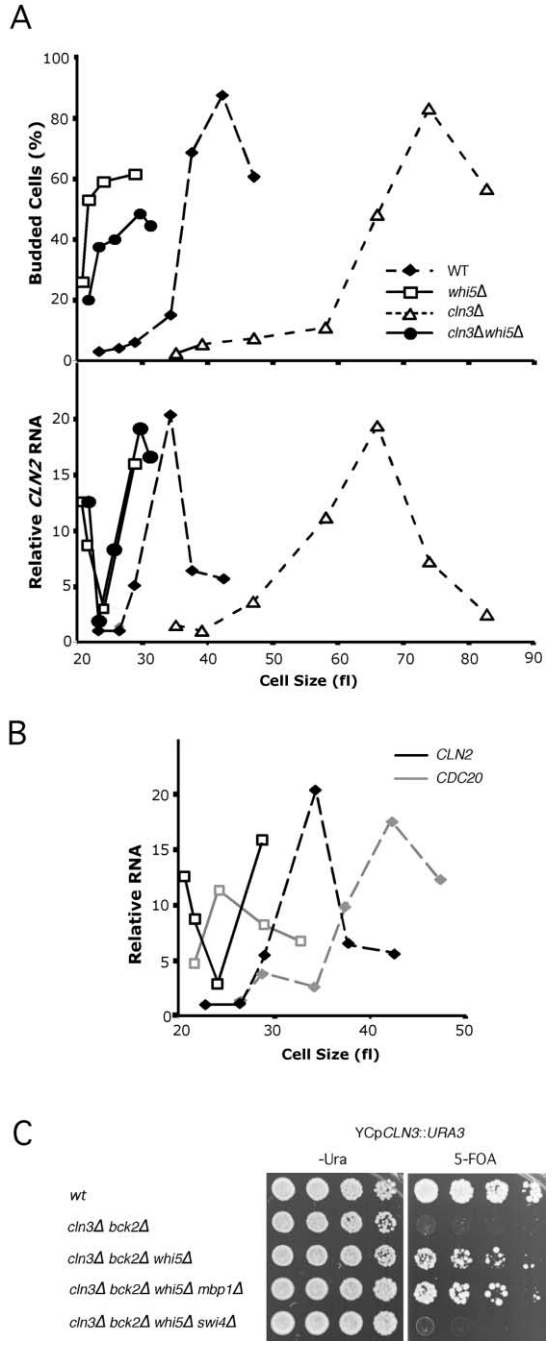

Figure 3. Cln3 Acts via Whi5 to Activate SBF-Dependent Cell Cycle Initiation

(A) *whi5*Δ mutants progress through Start independent of Cln3. (Top panel) Small unbudded cells were isolated by centrifugal elutriation, inoculated into fresh medium, and allowed to progress synchronously through the cell cycle. Cell size and budding index were determined at 20′ intervals. (Bottom panel) *CLN2* RNA levels were determined in the synchronized cells by RT-PCR. The transcript levels are expressed as the fold increase relative to the lowest level observed in the wild-type time course after normalization of all values to the *ACT1* RNA level in the same samples.

(B) Comparison of *CLN2* and *CDC20* RNA levels in wild-type and *whi5*Δ mutants. *CDC20* RNA levels were determined in the synchronized cells by RT-PCR and plotted along with the data for *CLN2* RNA presented in the bottom panel of (A). Lines and symbols used are consistent with those used in (A).

that *whi5*Δ results in a dramatic reduction in cell size in both wild-type (Figure 2A) and *mbp1*Δ mutant strains (data not shown) but does not affect the size of *swi4*Δ mutants (Figure 2A), consistent with the hypothesis that Whi5 negatively regulates SBF but not MBF. Furthermore, as expected, expression of *GAL1-WHI5* results in an increase in cell size in otherwise wild-type cells (Figure 2B) but has little or no effect on *swi4*Δ mutants, which are already large (data not shown). Together, these observations suggest that the inhibitory effect of Whi5 is exerted exclusively via SBF. In fact, simply integrating a second allele of *WHI5* in tandem with the wild-type allele results in a significant increase in cell size (data not shown), suggesting that *WHI5* acts as a dose-dependent inhibitor of Start, much like *CLN3* acts as a dose-dependent activator (Wijnen et al., 2002).

Cln3 Acts via Whi5 to Promote Cell Cycle Initiation

To evaluate whether Cln3 antagonizes Whi5 activity, we compared the cell size of asynchronous populations of *whi5*Δ and *cln3*Δ mutants to *cln3*Δ *whi5*Δ mutants (Figure 2C). Inactivation of Whi5 was not only sufficient to suppress the increase in cell size caused by inactivation of Cln3 but resulted in a cell size distribution identical to that caused by *whi5*Δ. This epistasis relationship suggests that Cln3 acts via Whi5 to regulate cell cycle initiation.

To eliminate the potential contributions to cell size of cell cycle defects outside of G1 phase, we evaluated the critical cell size (cell volume) at which bud emergence occurs in small G1 daughter cells isolated by centrifugal elutriation. Previous analysis has shown critical cell size at budding to be directly related to the activation of G1-specific transcription and to be a sensitive assay for the G1-specific function of Cln3 (Dirick et al., 1995; Stuart and Wittenberg, 1995). Small G1 daughter cells were isolated from asynchronous populations of wild-type and *whi5*Δ strains by centrifugal elutriation and allowed to progress through the cell cycle. Whereas wild-type cells budded at ~35 fl, *whi5*Δ resulted in a dramatic reduction in the cell size required for budding (<20 fl; Figure 3A, top panel). In fact, isolation of the smallest cells from the asynchronous population failed to yield greater than 80% unbudded cells, many of which budded within the first 20 min of growth. We conclude that the *whi5*Δ mutation results in a dramatic advancement of Start and that a large fraction of the small G1 daughter cells in the *whi5*Δ population have already executed Start prior to abscission from the mother cell.

To more precisely establish the relationship between Whi5 and Cln3, we determined the effect of *whi5*Δ on the timing of cell cycle initiation in *cln3*Δ mutants. As shown previously, *cln3*Δ mutants bud at almost twice the size of wild-type cells (~60 fl; Figure 3A, top panel) (Cross, 1988; Nash et al., 1988), consistent with the dependency upon Cln3 for cell cycle initiation during G1 phase. Strikingly, *cln3*Δ *whi5*Δ double mutants bud at

(C) *whi5*Δ bypasses the lethality caused by *bck2*Δ *cln3*Δ. (Left panel) Growth of the indicated strain carrying YCp*CLN3::URA3* under selective conditions. (Right panel) Growth of the same strains on 5-FOA to select against the YCp*CLN3::URA3* plasmid.

a comparable size to *whi5*Δ mutants (<20 fl; Figure 3A, top panel). As with *whi5*Δ mutants, it is difficult to isolate a pure unbudded population. Both of these observations demonstrate that *whi5*Δ is epistatic to *cln3*Δ, suggesting a pathway in which Cln3 exerts its positive effect on cell cycle initiation by antagonizing the negative effect of Whi5.

Whi5 Represses G1-Specific Transcriptional Activation Upstream of SBF

The early initiation of budding, as observed in *whi5*Δ mutants, often reflects premature activation of G1-specific transcription. To evaluate SBF-dependent transcription, we measured the level of transcripts from *CLN2*, a well-characterized SBF-dependent gene (Figure 3A, bottom panel). The level of the *CLN2* transcript in wild-type cells peaks at 35 fl, corresponding with bud emergence, and is subsequently rapidly lost, presumably due to transcriptional inactivation associated with cell cycle progression (Amon et al., 1993). In contrast, the *CLN2* transcript is high in the smallest *whi5*Δ mutant cells and, as observed in wild-type cells, rapidly decreases as budding increases to the maximum level. We believe this unexpected behavior to be a consequence of our failure to isolate a *whi5*Δ population in which the majority of cells are pre-Start and, therefore, undergoing transcriptional activation of G1-specific genes. Consistent with that interpretation, the inactivation of G1-specific transcription observed during the first cell cycle in the *whi5*Δ population is followed by the accumulation of the *CDC20* transcript (solid gray line, Figure 3B), which accumulates only after cells have passed Start and entered the budded phase of the cell cycle in wild-type cells (dashed gray line, Figure 3C) (Spellman et al., 1998). *CDC20* expression decreases when a second peak of accumulation of the *CLN2* transcript is observed.

Consistent with its effect on the minimal cell size at budding, inactivation of *CLN3* in wild-type cells results in a dramatic delay in transcriptional induction of *CLN2* (Figure 3A, bottom panel). Analysis of the *CLN2* transcript in a *whi5*Δ *cln3*Δ double mutant revealed that, like bud emergence, *CLN2* expression is coincident with that observed in the strain carrying *whi5*Δ alone (Figure 3A, bottom panel).

Together, these findings demonstrate that inactivation of *whi5*Δ bypasses the requirement for Cln3 for SBF-dependent transcriptional activation. We conclude that Whi5 delays G1-specific transcriptional activation and thereby cell cycle initiation and that Cln3 antagonizes that inhibition.

Inactivation of Whi5 Bypasses the Lethality of *bck2*Δ *cln3*Δ Mutants

Because inactivation of *WHI5* was sufficient to bypass the requirement for *CLN3* for activation of SBF-dependent transcription, we sought to determine whether it could also bypass the lethality resulting from *bck2*Δ *cln3*Δ (Figure 3C). Whereas a *bck2*Δ *cln3*Δ mutant is unable to survive under conditions that select for loss of the YEp*URA3-CLN3* plasmid, the *bck2*Δ *cln3*Δ *whi5*Δ mutant remains viable following loss of that plasmid. To determine whether SBF, MBF, or both are required for that bypass, we evaluated the requirement for *SWI4* and *MBP1* (Figure 3C). While *bck2*Δ *cln3*Δ *whi5*Δ *mbp1*Δ mutants are able to proliferate in the absence of YEp*URA3-CLN3*, *bck2*Δ *cln3*Δ *whi5*Δ *swi4*Δ mutants are not. Thus, bypass of the lethality of *bck2*Δ *cln3*Δ mutants depends upon the activity of SBF. Consistent with this finding, the level of *CLN2* gene expression in an asynchronous population of *bck2*Δ *cln3*Δ *whi5*Δ mutants is comparable to that observed in wild-type cells, whereas expression is negligible in a *bck2*Δ *cln3*Δ (*GAL1-CLN3*) strain grown on glucose (data not shown). In contrast, we find that inactivation of *WHI5* is insufficient to support the viability of either *bck2*Δ *swi6*Δ *GAL1-CLN2* or *cln1*Δ *cln2*Δ *cln3*Δ *GAL1-CLN3* strains in the presence of glucose (data not shown). These data provide additional support for the SBF-specific role of Whi5 and, more importantly, show that inactivation of *WHI5* abolishes the requirement for the G1-specific transcriptional activators *CLN3* and *BCK2*.

Whi5 Binds via SBF to Promoters of Transcriptionally Inactive G1-Specific Genes

The identification of Whi5 as an SBF bound inhibitor of transcriptional activation suggests that it may act directly at the level of SBF-dependent promoters. Binding of epitope-tagged Swi4, Swi6, and Whi5 to two G1-specific promoters, *CLN2* (SBF specific) and *CLB5* (MBF specific), and the *HXT3* promoter (SBF/MBF independent) was assessed by ChIP analysis. Like Swi4 (Cosma et al., 2001; Cross et al., 1994; Koch et al., 1996), Whi5 binds to the *CLN2* promoter (Figure 4A) and only weakly, or not at all, to the *CLB5* promoter. As previously demonstrated, Swi6 binds efficiently to both of the *CLN2* and *CLB5* promoters (Harrington and Andrews, 1996; Koch et al., 1996), whereas none of those proteins binds detectably to the *HXT3* promoter (Figure 4A and data not shown). Binding of Whi5 to the *CLN2* promoter is not observed in a *swi4*Δ strain (Figure 4B), and, as predicted, that binding is independent of Mbp1. We conclude that Whi5 binds to G1-specific promoters via SBF.

To evaluate the relationship between Whi5 binding to SBF-dependent promoters and G1-specific transcriptional activation, we analyzed timing of Whi5 binding to the promoters of three SBF targets, *CLN2*, *CLN1*, and *PCL1*, by ChIP analysis following release from G1 phase arrest by mating pheromone. We find that, like Swi6, Whi5 is bound to the *CLN2* promoter in mating pheromone-arrested cells. However, in contrast to Swi6, it dissociates just prior to transcriptional activation (Figure 4C, upper panel). As indicated by Swi6 binding, SBF remains associated with the *CLN2* promoter until cells enter the budded phase of the cell cycle and transcription is inactivated (Figure 4C, lower panel). A similar pattern of Whi5 binding was observed at the *CLN1* and *PCL1* promoters. Thus, association of Whi5 with SBF-dependent promoters early in G1 phase correlates with transcriptional repression, and its subsequent dissociation correlates with Cln3/CDK-dependent G1-specific transcriptional activation.

We evaluated whether Cln3 was sufficient for dissociation from SBF-dependent promoters or whether additional cyclins were required. Binding of Whi5 to the same three promoters was evaluated by chromatin immunoprecipitation in cells that lack *CLN3*, express *CLN3* as

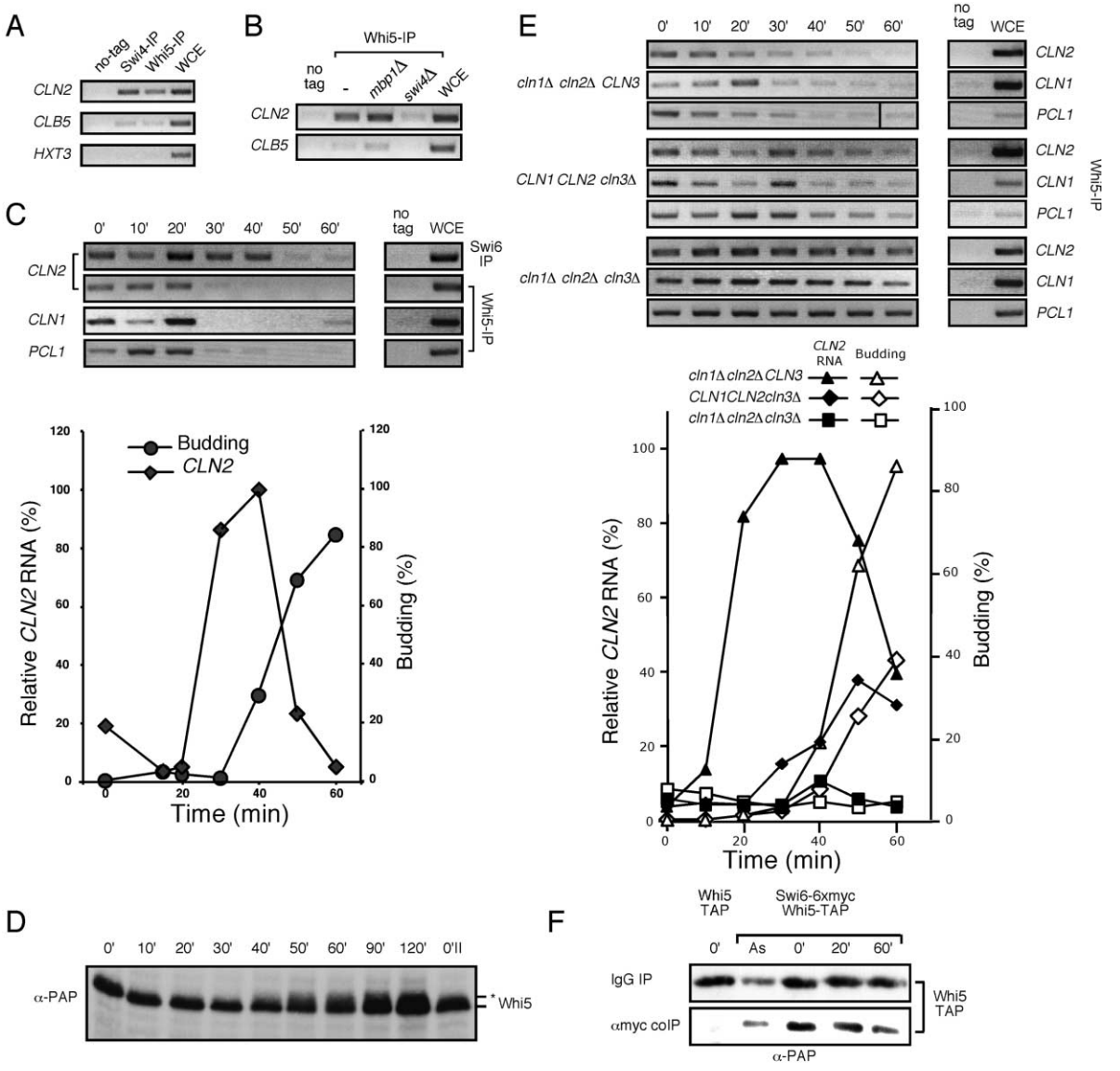

Figure 4. Whi5 Hyperphosphorylation Correlates with Disassociation of Whi5 from SBF Bound to Transcriptionally Active G1-Specific Gene Promoters

(A) Whi5 binds to *CLN2* promoter DNA. Chromatin immunoprecipitation of *CLN2* (SBF dependent), *CLB5* (MBF dependent), and *HXT3* (SBF/MBF independent) promoter DNA by Swi4-myc and Whi5-myc immunoprecipitation. Immunoprecipitation from a strain containing no epitope-tagged genes is presented as a negative control.

(B) Whi5 binds *CLN2* promoter via SBF. Chromatin immunoprecipitation of *CLN2* and *CLB5* promoter DNA from a wild-type (wt), *mbp1*Δ, or *swi4*Δ strain by Whi5-myc immunoprecipitation.

(C) Whi5 disassociation from *CLN2* promoter correlates with transcription activation of SBF-dependent promoters. (Top panel) Chromatin immunoprecipitation of the *CLN2* promoter DNA by Swi6-myc and of *CLN1, CLN2*, and *PCL1* promoter DNA by Whi5-myc from cells arrested by α factor (0′) or released from the arrest for the indicated interval (10′–60′). (Bottom panel) Budding index and *CLN2* RNA levels in cells from the same time course.

(D) Slower-migrating form of Whi5 accumulates during late G1 phase. Western blot analysis of Whi5 protein from samples taken during the α factor arrest and release time course shown in (C).

(E) Whi5 disassociation from SBF-dependent promoters is Cln3 dependent. (Top panel) Chromatin immunoprecipitation of *CLN2*, *CLN1*, and *PCL1* promoter DNA by Whi5-myc immunoprecipitation from *cln1*Δ *cln2*Δ*xs CLN3*, *CLN1 CLN2 cln3*Δ, and *cln1*Δ *cln2*Δ*xs cln3*Δ *TRP1::GAL1-CLN3* (*cln*-less) strains during α factor arrest and release in glucose-containing medium. (Bottom panel) Budding index and *CLN2* RNA levels in the same samples.

(F) Whi5 binds SBF throughout the cell cycle. Coimmunoprecipitation of Swi6-myc in Whi5-TAP immunecomplexes from asynchronous cells (As) or following α factor arrest (0′) and release for the indicated intervals (20′ and 60′).

their only G1 cyclin, or express no G1 cyclins. As predicted, a decrease in the binding of Whi5 to all three promoters correlated with the activation of G1-specific transcription (Figure 4E). In cells lacking G1 cyclins, *CLN2* expression remained repressed, and Whi5 remained bound to the *CLN2* promoter. In cells having

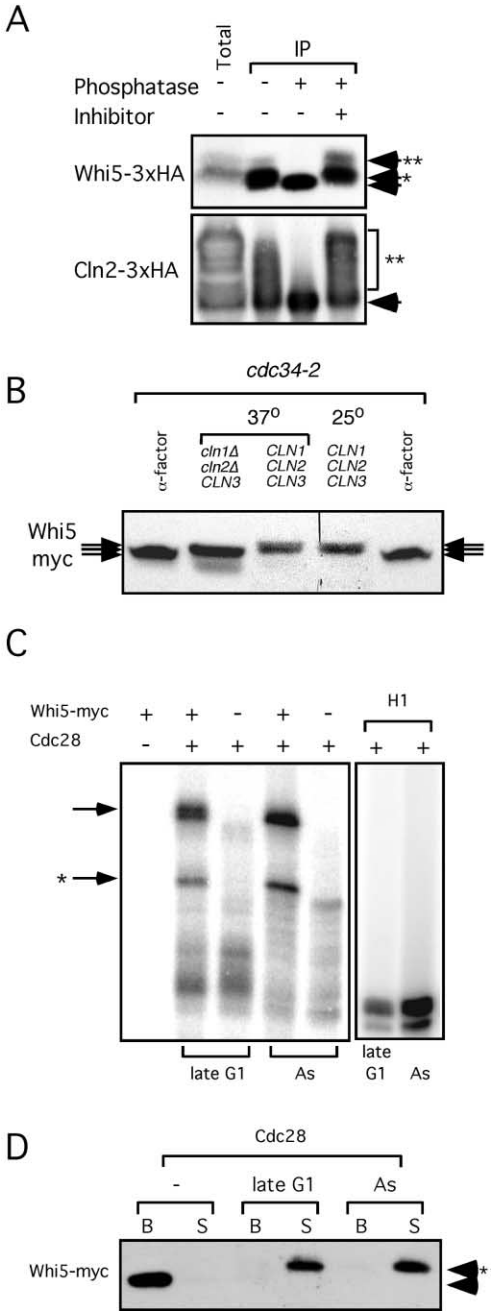

Figure 5. Phosphorylation of Whi5 by G1 Cyclin-Dependent Kinase Cdc28 Promotes Disassociation from SBF In Vitro

(A) Whi5 is hypo- and hyperphosphorylated in vivo. Western blot analysis of Whi5-HA and Cln2-HA in whole cell extract (total) or following immunoprecipitation and treatment with lambda phosphatase in the presence or absence of phosphatase inhibitors. Arrows indicate dephosphorylated, hypophosphorylated (*), and hyperphosphorylated (**) protein migration.

(B) Phosphorylation of Whi5 by various forms of the Cdc28 CDK. Extracts were prepared from *cdc34-3* or *cdc34-3 cln1*Δ *cln2*Δ*xs* strains expressing Whi5-13xmyc. Cells were either arrested during G1 by α factor arrest, arrested during late G1 by *cdc34-3* at the restricted temperature (37°C) for 180 min, or grown at the permissive temperature (25°C) for 180 min after release from α factor arrest to allow progression out of G1 phase. Arrows indicate different mobility forms of Whi5-13xmyc protein. Signal in the *CLN1 CLN2 CLN3* lanes at 37°C and 25°C was digitally enhanced to facilitate the visualization of the differing mobility.

only *CLN3* (Figure 4E), the rapid activation of *CLN2* expression following release from arrest was coordinate with the dissociation of Whi5 from each of the SBF-dependent promoters, as observed in wild-type cells. In contrast, cells lacking *CLN3* only activated *CLN2* expression after a significant delay and did so more gradually than either wild-type or *cln1*Δ *cln2*Δ cells. Consistent with the pattern of expression, a marked decrease in the extent and rate of dissociation of Whi5 was observed. Whereas dissociation appears rapid and concerted in the wild-type strain (Figure 4C), the decrease is more gradual in the *cln1*Δ *cln2*Δ strain (Figure 4E), suggesting a contribution of other G1 cyclins. Nevertheless, *CLN1* and *CLN2* are insufficient in the absence of *CLN3*, since Whi5 appears to remain bound in that strain until late in the time course. Together, these data strongly suggest that transcriptional activation via Cln3 is exerted via its capacity to antagonize Whi5 binding to G1-specific promoters.

Whereas the dissociation of Whi5 from SBF bound promoters at the time of transcriptional activation is consistent with its behavior as a transcriptional repressor of SBF-dependent genes, it does not explain the results of our MudPIT analysis that shows that Whi5 is present in a complex with SBF from cells actively transcribing *CLN2*. To address this issue, we analyzed Whi5 binding to Swi6 in synchronized cells by coimmunoprecipitation (Figure 4F). Whi5 was found in a complex with Swi6 even subsequent to the dissociation of both proteins from promoters. We conclude that Whi5 forms a complex with SBF independent of its binding to G1-specific promoters.

Whi5 Hyperphosphorylation Is Associated with G1-Specific Transcriptional Activation

Our data suggest that Whi5 might be a direct target for phosphorylation by Cln3-associated CDK. Several minor species of reduced mobility are apparent in HA-tagged Whi5 from asynchronous cells (Figure 5A). Consistent with their identity as phosphorylated species, treatment of immune complexes containing Whi5-HA with lambda phosphatase resulted in conversion of those spe-

(C) Whi5 is a target of cyclin-dependent kinase Cdc28 in vitro. (Left panel) Full-length Whi5-13xmyc prepared under denaturing conditions was used as a substrate for phosphorylation by purified Cdc28-TAP complexes from asynchronous cells (As) and *cdc34-3* mutant cells synchronized during late G1 phase by release from an α factor arrest at the restrictive temperature (37°C) for 60 min (late G1). Arrows indicate phosphorylated Whi5-myc species. The mobility of the full-size Whi5-13xmyc protein and a degradation product (*) is indicated. (Right panel) Protein kinase activity of purified soluble Cdc28 CDK was determined using histone H1 as a substrate. Histone H1 (1 μg) was used in each kinase assay. The exposure shown for histone H1 is 1/100 of that shown for Whi5.

(D) Whi5 disassociates from SBF complex when phosphorylated by cyclin-dependent kinase Cdc28. Calmodulin beads containing TAP-purified SBF prepared from cells expressing Swi4-TAP and Whi5-13xmyc and arrested during G1 by α factor were treated with soluble purified Cdc28 CDK complexes from either asynchronous cells (As) or late G1 cells as indicated in (C). Following treatment, the calmodulin bead bound fraction (B) or the fraction released into the supernatant (S) were analyzed by immunoblotting with anti-myc antibody. Arrows indicate different mobility forms of Whi5-13xmyc protein.

cies to a single species of higher mobility than those observed in untreated samples or in samples treated with phosphatase in the presence of phosphatase inhibitors. The well-characterized phosphoprotein Cln2 is provided as a positive control (Lanker et al., 1996).

To determine the relationship between phosphorylation of Whi5 and its disassociation from G1-specific promoters, we examined the mobility of the Whi5 protein in the same population of synchronized cells used for the ChIP analysis of Swi6 (Figure 4D). Whereas Whi5-TAP migrates as a single species during early G1 phase (Figure 4C), a slower-migrating form of Whi5-TAP appears and becomes increasingly apparent as cells progress into the budded phase of the cell cycle. Phosphatase treatment of Whi5-TAP demonstrates that, like the Whi5-HA protein shown in Figure 5A, even the highest-mobility species is phosphorylated (data not shown). We conclude that Whi5 is phosphorylated at one or more positions during all cell cycle phases but that a portion of that protein becomes hyperphosphorylated as cells progress through G1 phase.

To establish the form of the CDK responsible for Whi5 phosphorylation, we compared the mobility of Whi5 in cells in which specific cyclins accumulate. Whereas all three G1 cyclins accumulate in a *cdc34-ts* strain arrested during late G1 phase at the restrictive temperature, a *cln1*Δ *cln2*Δ *cdc34-ts* strain accumulates only Cln3. B-type cyclins fail to accumulate due to the inability of *cdc34* mutants to degrade the Clb/CDK inhibitor Sic1 (Schwob and Nasmyth, 1993). Those strains were arrested in late G1 phase by shifting to the restrictive temperature for 3 hr, and the electrophoretic mobility of Whi5 was compared to Whi5 from the same cells either arrested by α factor to inactivate all forms of the CDK or allowed to progress into G2/M phase (Figure 5B). That analysis revealed that, whereas Cln3/CDK promotes only a small shift in mobility, the accumulation of all three G1 cyclins results in more extensive retardation, similar to that observed at the permissive temperature where B-type cyclin-associated forms of the CDK accumulate. These data suggest that Cln3/CDK promotes quantitative phosphorylation of Whi5, then leads to progressively greater phosphorylation by Cln1- and Cln2-associated CDK and then by Clb/CDK. This is consistent with the effect of Cln3 activity on Whi5 binding to G1 specific promoters and with the finding that dissociation of Whi5 from those promoters is less concerted in the absence of *CLN1* and *CLN2* (Figure 4E).

Whi5 Phosphorylation by Cdc28 Kinase Promotes Dissociation from SBF In Vitro

We evaluated whether Whi5 could act as a target for the Cdc28 CDK in vitro. TAP-tagged Cdc28 was purified from either asynchronous cells or cells expressing only G1 cyclins (*cdc34-ts* mutants arrested following release from α factor arrest). Both preparations of Cdc28 CDK kinase were active against histone H1 and immunoprecipitated 13xmyc-tagged Whi5 (Figure 5C). Based upon their relative kinase activity, measured using histone H1 as a substrate, the Cln-associated CDK appeared to be more efficient than the total CDK at phosphorylating Whi5. No phosphorylation of Whi5 was observed when the immune complexes were prepared from a strain expressing untagged Whi5 or when no Cdc28 CDK was added. Thus, Whi5 is a substrate of the Cdc28 CDK in vitro. This agrees with a recent study (Ubersax et al., 2003), in which Whi5 was detected among the budding yeast proteins that are directly phosphorylated by the Cdc28 CDK in vitro.

To evaluate whether phosphorylation by the Cdc28 CDK promotes dissociation of Whi5 from SBF in vitro, we prepared SBF complexes containing Whi5-myc by purification of Swi4-TAP from cells arrested during G1 by depletion of G1 cyclins. The Whi5-SBF complexes retained on calmodulin beads were treated with either Cln-CDK complexes or Cdc28 CDK from asynchronous cells (Figure 5D). In both cases, Whi5-myc was phosphorylated and released into the supernatant, whereas it was retained on the beads in the untreated samples. We conclude that Cln-CDK-dependent phosphorylation is sufficient to promote dissociation of Whi5 from SBF, consistent with the timing of its dissociation from promoters in vivo.

Phosphorylation of Whi5 Determines the Timing of SBF-Dependent Transcriptional Activation and Cell Cycle Progression

Whi5 contains four perfect CDK consensus sites (S/T-P-X-K/R; sites 1, 2, 11, and 12) and eight minimal consensus sequences (S/T-P; sites 3–10; Figure 6A). Analysis of TAP-purified Whi5 obtained from transcriptionally active G1 phase cells by mass spectrometry revealed the presence of phosphopeptides representing five putative CDK sites (sites 2, 4, 5, 10, and 12; data not shown). We mutated the phosphoacceptor amino acid at those sites, along with two other sites (sites 8 and 9), to alanine. Surprisingly, we were unable to introduce the mutant gene (designated *WHI5^{7A}*) under control of the endogenous *WHI5* promoter into cells, either alone or in tandem with a wild-type copy of the gene. The basis for that lethality has not been established. To obviate that problem, the mutant and wild-type open reading frame were placed under control of the conditional *MET3* promoter and integrated into the genome of *whi5*Δ mutant cells. As expected, both strains exhibited the small cell size phenotype of the *whi5*Δ mutant when grown on methinonine-containing medium (+Met) to repress *MET3*-dependent transcription. Although cells expressing either *MET3-WHI5* or *MET3-WHI5^{7A}* became larger than wild-type cells after the shift to methionine-deficient medium (−Met) for several generations, the increase in cell size observed in the strain expressing *WHI5^{7A}* was substantially greater (data not shown). Analysis of Whi5-myc expressed from the *MET3* promoter revealed that the level of expression of both Whi5 and Whi5^{7A} was comparable to that expressed from the endogenous promoter (Figure 6B). This suggests that the effect of expression of *MET3-WHI5* might result from misregulation of *WHI5* expression rather than from overexpression.

To evaluate the importance of phosphorylation during G1 phase, cells expressing either *MET3-WHI5* or *MET3-WHI5^{7A}* were pregrown in +Met medium and shifted to −Met medium to induce the *MET3* promoter, and then small cells were isolated by centrifugal elutriation and evaluated for cell size, G1-specific gene expression,

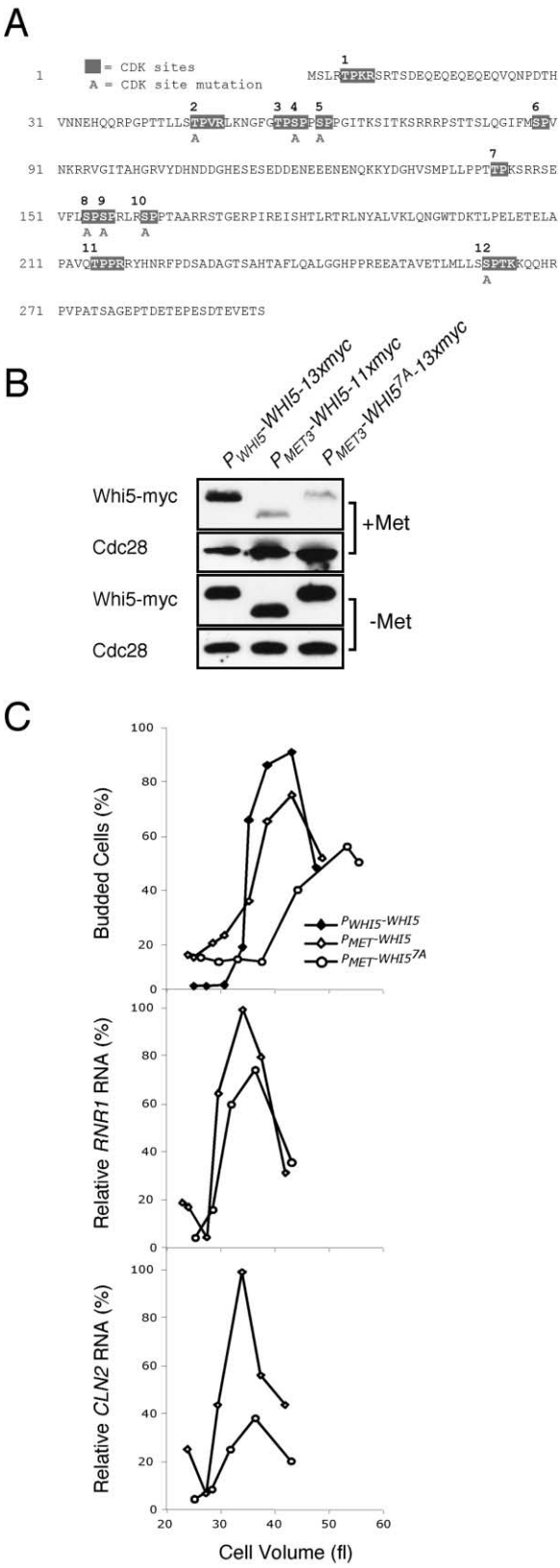

Figure 6. Whi5 Phosphorylation at CDK Consensus Sites Is Required for Efficient Transcriptional Activation of SBF-Dependent Genes and, Consequently, for Timely Cell Cycle Initiation

(A) Putative CDK phosphorylation sites in Whi5. CDK sites are shown in gray. Sites mutated in this study are indicated by the amino acid substituted with alanine (A).

(B) Whi5 protein expressed from the inducible *MET3* promoter accumulates to a similar level to wild-type Whi5. Western blot analysis of Whi5 protein isolated from asynchronous cells expressing *WHI5* from the endogenous promoter or *WHI5* or *WHI5^{7A}* from the *MET3* promoter under repressing (+Met) or inducing (−Met) conditions. Cdc28 is presented as loading control.

(C) Mutation of CDK phosphorylation sites in Whi5 delays cell cycle initiation and reduces and delays SBF-dependent transcriptional activation. Cells were grown in −Met medium for 25′ prior to centrifugal elutriation to induce *WHI5* and *WHI5^{7A}* transcription. Small unbudded cells were isolated by centrifugal elutriation, inoculated into fresh −Met medium, and allowed to progress synchronously through the cell cycle. (Top panel) Cell size, budding index and RNA levels were determined at 20′ intervals. (Middle panel) *RNR1* RNA levels determined in the synchronized cells by RT-PCR. (Bottom panel) *CLN2* RNA levels determined by RT-PCR. The transcript levels are presented as a percentage relative to the highest level observed in both time courses after normalization of all values to the *ACT1* RNA level in the same samples.

and budding (Figure 6C). As expected, both the timing and extent of transcriptional activation and budding observed in the *MET-WHI5* strain were comparable to wild-type cells. In contrast, the *MET-WHI5^{7A}* mutant budded at a substantially larger cell size, and the expression of the SBF-dependent *CLN2* transcript was delayed and reduced relative to that in cells expressing wild-type *WHI5*. Consistent with the specificity of Whi5 for SBF, the MBF-dependent transcript *RNR1* was expressed at a comparable level to cells expressing mutant or wild-type *WHI5*. Finally, the lower-mobility phosphorylated forms of Whi5 observed following release from α factor arrest in wild-type cells (Figure 4D) and cells expressing *MET-WHI5-myc* fail to accumulate in the *MET-WHI5^{7A}-myc* mutant (data not shown). We conclude that phosphorylation of Whi5 at one or more sites affected by the CDK site mutations is required for efficient and timely activation of SBF-dependent genes.

Discussion

We have shown that Whi5 interacts specifically with SBF but not independently with either of its known subunits or MBF. Whi5 binds to SBF at G1-specific promoters and acts as a repressor of G1-specific transcriptional activation. As cells progress into late G1 phase, Whi5 dissociates from promoters in a Cln3-dependent manner (Figure 7A). Dissociation is promoted by and dependent upon Cln3 in vivo and is promoted by Cln/CDK in vitro. Consistent with its behavior as an in vitro substrate for the Cdc28 CDK, mass spectrometric analysis of Whi5 demonstrates that it is phosphorylated in vivo at CDK consensus sites during late G1 phase (data not shown). Mutational analysis of a subset of the putative CDK sites in Whi5 demonstrates that Whi5 phosphorylation is required for efficient transcriptional activation of SBF-dependent genes and, consequently, for timely cell cycle initiation.

Whi5 is not simply required to keep SBF-dependent gene expression low outside of G1 phase but acts as a component of the mechanism governing periodic transcription. The pattern and extent of transcriptional activation of *CLN2* in *whi5*Δ and *cln3*Δ*whi5*Δ mutants is

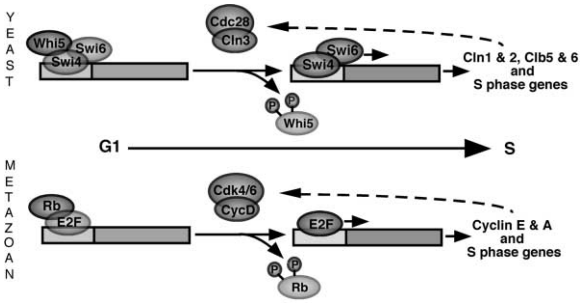

Figure 7. Model for Regulation of G1-Specific Transcriptional in Yeast and Mammalian Cells

(A) Cln3-dependent CDK phosphorylates Whi5, releasing SBF from negative regulation. Activation of SBF-dependent genes promotes S phase entry and leads to expression of *CLN1* and *CLN2*. Accumulation of Cln1-2/CDK further phosphorylates Whi5.

(B) Cyclin D/CDK phosphorylates Rb, releasing E2F from negative regulation. Activation of E2F-responsive genes promotes S phase entry and leads to expression of cyclin E. Accumulation of cyclin E/CDK further phosphorylates Rb, contributing to the activation of E2F-responsive genes.

indistinguishable, arguing that *CLN3* plays no role in SBF-dependent transcription under those conditions in the absence of Whi5. Whi5 is not necessary for repression of transcription outside of G1 phase. Overexpression of Whi5 or elimination of CDK phosphorylation sites leads to large cell size and delayed transcriptional activation, whereas inactivation of Whi5 leads to small cell size and early transcription. Thus, inactivation of Whi5 is both necessary and sufficient for activation of transcription and therefore is sufficient to explain the requirement for *CLN3*. Timely dissociation of Whi5 from SBF-dependent promoters depends upon *CLN3* and is coincident with transcriptional activation.

Whi5 persists outside of G1 phase and is found in association with SBF subsequent to SBF disassociation from promoters. Whether these complexes fail to associate with promoters due to their state of modification, their localization, alterations in their composition, or other properties remains to be established. However, the presence of Whi5, whether free or in a complex with SBF, places it in a position to play roles beyond its activity at promoters. For example, Whi5 may regulate localization of SBF or its assembly or association with promoters. Those additional functions might explain the effect of overexpression of Whi5 and perhaps even the lethality of the *WHI5*^{7A} mutant under the endogenous promoter.

Although inactivation of Whi5 occurs at the time of transcriptional activation of SBF-dependent genes and is sufficient for activation, additional mechanisms are certainly involved in the regulation of SBF-dependent and, more generally, G1-specfic transcription. Both SBF and MBF are targets for CDK-dependent phosphorylation and, as such, are likely targets of regulatory mechanisms that overlap with or perhaps act in concert with dissociation of Whi5 from the SBF complex. Because MBF- and SBF-dependent transcription are cocoordinately regulated, it has been assumed that this occurs via a conserved mechanism, likely involving the shared component Swi6. The specificity of Whi5 for SBF calls that assumption into question. In fact, there is evidence that transcriptional repression of SBF- and MBF-dependent genes subsequent to Start occurs via distinct mechanisms (Amon et al., 1993). A recent study shows that Stb1, an established Swi6 binding protein, specifically affects MBF-dependent transcriptional activation (Costanzo et al., 2003). Although it was found to specifically associate with MBF in that study, we identified Stb1 with a high degree of confidence by MudPIT analysis in both SBF- and MBF-containing samples (data not shown). Preliminary analysis is consistent with a role for Stb1 in addition to its role in MBF-dependent transcription (data not shown). However, its importance in wild-type cells and whether that role is SBF dependent are unclear.

Additional factors found by MudPIT analysis to interact specifically with SBF, MBF, Swi4, and Mbp1 are currently under investigation. Preliminary data show that several of the SBF/MBF interactors have an effect on cell size either when inactivated or overexpressed, consistent with effects on SBF- and MBF-dependent transcription. Efforts are underway to unravel the biological significance of those interactions.

The existence of an inhibitor of G1-specific transcription that is antagonized by Cln3/CDK has been hypothesized based upon analogy of metazoan Rb. The identification of Whi5 as such an inhibitor shows yet again that there is a close functional homology between the regulatory components involved in G1-specific transcriptional activation in yeast and mammalian cells. The similarities between Whi5 and Rb are striking (Figure 7). In mammalian cells, passage through the restriction point and entry into S phase is determined at least in part by the phosphorylation of Rb by cyclin D-Cdk4/Cdk6 and subsequently cyclin E-Cdk2 (reviewed by Stevens and La Thangue [2003]). In yeast, phosphorylation of Whi5 by Cln3/CDK, and possibly subsequently by Cln1-2/CDK, leads to transcriptional activation of SBF-responsive genes promoting passage through Start, the primary gating event of the yeast cell cycle. Cln3 and Cln1/2 have been equated to mammalian cyclin D1 and cyclin E, respectively (Hatakeyama et al., 1994). Furthermore, like the effect of Whi5 on G1-specific transcription factors in yeast, the effect of Rb association with E2F is only one of several pathways regulating E2F activity. It is clear that resolution of the seemingly complex network of transcriptional regulators involved in G1-specific gene regulation and, more generally, in cell cycle-dependent regulation of gene expression in both yeast and metazoans remains a major goal.

Experimental Procedures

Strains and DNAs

All yeast strains used in this study were derived from 15Daub (*MATa ade1 leu2-3,112 his2 trp1-1 ura3Δns bar1Δ*). The table of yeast stains used is presented in Supplemental Data (see Table S1 at http://www.cell.com/cgi/content/full/117/7/887/DC1). TAP strategy was carried out in CWY 747 (15Daub *TRP1::GAL1-CLN3 cln1Δ cln2Δ cln3Δ pep4::LEU2*). The strategy of Rigaut et al. (1999) was used to append a TAP tag to *SWI6, SWI4, MBP1, WHI5* (carboxyterminus), and *CDC28* (aminoterminus) at their endogenous loci. The PCR-based gene disruption method of Reid et al. (2002) was used to create the *cln3Δ bck2Δ* bypass strain. The PCR method of Longtine et al. (1998) was used to disrupt *WHI5*, *SWI4*, and *SWI6*, *GAL1*

promoter integrate, and 3xHA, 13xmyc, or 11xmyc tag at the carboxyterminus *WHI5*. 6xmyc-tagged alleles of *SWI4* and *SWI6*; disruption of *PEP4*, *MBP1*, and *SWI4*; 3xHA tagging of *MBP1*; and 6xHIS tagging of *SWI6* was done by plasmid integration. The *WHI5* ORF was cloned by PCR into the pCR 2.1-TOPO vector (Invitrogen). Phosphorylation site mutations were introduced by PCR, resulting in amino acid substitutions T47A, S59A, S62A, S155A, S157A, S161A, and S262A, mutating putative CDK sites 2, 4, 5, 8, 9, 10, and 12, respectively. The *WHI5* ORF, including 36 bp upstream sequence cloned into an integration plasmid containing the *MET3* promoter sequence, was integrated into the *URA3* locus.

TAP Affinity Purification and MudPIT Analysis

TAP purification and analysis was carried out as described by Boddy et al. (2001). MudPIT was carried out as described by McDonald and Yates (2002). Identification by MudPIT of potentially phosphorylated peptides was done as described (MacCoss et al., 2002).

Coimmunoprecipitation

Immunoprecipitations were carried out using TAP purification buffers. Immunoprecipitated proteins were resolved by 12.5% SDS-PAGE.

Cell Synchronization

The conditional *cln*-deficient strain (CWY747) was arrested during G1 phase by growth in YEP-raffinose and released into Start by addition of 2% galactose to induce expression of *GAL-CLN3* as described by Stuart and Wittenberg (1995). A population of small G1-synchronized cells was obtained by centrifugal elutriation as described by Stuart and Wittenberg (1995), except that the sonication step was omitted and cells were grown in YEPD throughout the experiment. Mating pheromone arrest synchrony experiments were also carried out as described by Stuart and Wittenberg (1995).

Analysis of Whi5 Phosphorylation

Phosphorylation analysis of the Whi5 protein was performed as described by Flick et al. (2003).

Real-Time RT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen). The QuantiTech SYBR Green RT-PCR Kit (Qiagen) was used for RT-PCR experiments. Reactions were run on the Smart Cycler II System (Cepheid) using standard RT-PCR conditions. Data were analyzed using Smart Cycler Software 2.0. The Lux Fluorogenic Primers (Invitrogen), used to detect *CDC20* and *ACT1* transcript levels with the SuperScript III Platinum One-Step RT-PCR System, were a kind gift of Bryan Olson, TSRI.

ChIP Analysis

Chromatin immunoprecipitation was performed as described by Flick et al. (2003).

In Vitro Kinase Assay

Soluble Cdc28 protein was purified from culture (YEPD containing 0.2 mM $CuSO_4$), expressing N-terminal TAP-tagged *CDC28* under the control of the *CUP1* promoter (Kesti et al., 2004) by the TAP purification method described by Boddy et al. (2001) from an asynchronize culture, and from cells, released from an α factor arrest and arrested during late G1 by *cdc34-3* at the restricted temperature (37°C) for 60 min. α-myc immunoprecipitate from α factor-arrested cells of Whi5 or 13xmyc-Whi5 fusion protein was used as substrate. Kinase assays were carried out as described by Deshaies and Kirschner (1995). Reactions were resolved by 12.5% SDS-PAGE. Phosphorylation of Whi5 was analyzed using a PhosphorImager (Molecular Diagnostics). In vitro kinase assay on SBF was carried out as described above. SBF was prepared by TAP purification from cells expressing Swi4-TAP and Whi5-myc arrested during G1 by mating pheromone. Phosphorylation and disassociation from SBF of Whi5-myc was analyzed by anti-myc Western blot.

Other Methods

Cell size analysis was performed using a Coulter Z2 Particle Cell Analyzer (Beckman-Coulter). Cell size distribution was analyzed using the Z2 AccuComp software (Beckman-Coulter).

Acknowledgments

The authors would like to thank Marisela Guaderrama for technical support; and Tapio Kesti, Karin Flick, David Stuart, Bryan Olson, and Fred Cross for materials used in this study. We thank Tapio Kesti, Nick Boddy, Karin Flick, Peter Kaiser, and Steve Reed for comments on the manuscript; and members of the TSRI Cell Cycle Group for helpful discussion. This work was supported by USPHS grant GM59941 to C.W.

Received: December 19, 2003
Revised: April 21, 2004
Accepted: April 22, 2004
Published: June 24, 2004

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.

Boddy, M.N., Gaillard, P.H., McDonald, W.H., Shanahan, P., Yates, J.R., III, and Russell, P. (2001). Mus81-Eme1 are essential components of a Holliday junction resolvase. Cell *107*, 537–548.

Breeden, L.L. (2003). Periodic transcription: a cycle within a cycle. Curr. Biol. *13*, R31–R38.

Cho, R.J., Campbell, M.J., Winzeler, E.A., Steinmetz, L., Conway, A., Wodicka, L., Wolfsberg, T.G., Gabrielian, A.E., Landsman, D., Lockhart, D.J., and Davis, R.W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. Mol. Cell *2*, 65–73.

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 Saccharomyces cerevisiae by the Swi6-binding protein Stb1. Mol. Cell. Biol. *23*, 5064–5077.

Cross, F.R. (1988). DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of Saccharomyces cerevisiae. Mol. Cell. Biol. *8*, 4675–4684.

Cross, F.R., Hoek, M., McKinney, J.D., and Tinkelenberg, A.H. (1994). Role of Swi4 in cell cycle regulation of CLN2 expression. Mol. Cell. Biol. *14*, 4779–4787.

Deshaies, R.J., and Kirschner, M. (1995). G1 cyclin-dependent activation of p34CDC28 (Cdc28p) in vitro. Proc. Natl. Acad. Sci. USA *92*, 1182–1186.

Di Como, C.J., Chang, H., and Arndt, K.T. (1995). Activation of CLN1 and CLN2 G1 cyclin gene expression by BCK2. Mol. Cell. Biol. *15*, 1835–1846.

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

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. Genes Dev. *12*, 2245–2262.

Epstein, C.B., and Cross, F.R. (1994). Genes that can bypass the CLN requirement for Saccharomyces cerevisiae cell cycle START. Mol. Cell. Biol. *14*, 2041–2047.

Flick, K., Chapman-Shimshoni, D., Stuart, D., Guaderrama, M., and Wittenberg, C. (1998). Regulation of cell size by glucose is exerted via repression of the CLN1 promoter. Mol. Cell. Biol. *18*, 2492–2501.

Flick, K.M., Spielewoy, N., Kalashnikova, T.I., Guaderrama, M., Zhu, Q., Chang, H.C., and Wittenberg, C. (2003). Grr1-dependent inactivation of Mth1 mediates glucose-induced dissociation of Rgt1 from HXT gene promoters. Mol. Biol. Cell *14*, 3230–3241.

Gallego, C., Gari, E., Colomina, N., Herrero, E., and Aldea, M. (1997). The Cln3 cyclin is down-regulated by translational repression and

degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. EMBO J. *16*, 7196–7206.

Gavin, A.C., and Superti-Furga, G. (2003). Protein complexes and proteome organization from yeast to man. Curr. Opin. Chem. Biol. *7*, 21–27.

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.

Ho, A., and Dowdy, S.F. (2002). Regulation of G(1) cell-cycle progression by oncogenes and tumor suppressor genes. Curr. Opin. Genet. Dev. *12*, 47–52.

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

Iyer, V.R., Horak, C.E., Scafe, C.S., Botstein, D., Snyder, M., and Brown, P.O. (2001). Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. Nature *409*, 533–538.

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

Kesti, T., McDonald, W.H., and Yates, J.R., III, and C., W. (2004). Cell cycle-dependent phosphorylation of the DNA polymerase epsilon subunit, Dpb2, by the Cdc28 cyclin-dependent protein kinase. J. Biol. Chem. *279*, 14,245–14,255.

Koch, C., Moll, T., Neuberg, M., Ahorn, H., and Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. Science *261*, 1551–1557.

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.

Lanker, S., Valdivieso, M.H., and Wittenberg, C. (1996). Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. Science *271*, 1597–1601.

Longtine, M.S., McKenzie, A., III, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast *14*, 953–961.

MacCoss, M.J., McDonald, W.H., Saraf, A., Sadygov, R., Clark, J.M., Tasto, J.J., Gould, K.L., Wolters, D., Washburn, M., Weiss, A., et al. (2002). Shotgun identification of protein modifications from protein complexes and lens tissue. Proc. Natl. Acad. Sci. USA *99*, 7900–7905.

MacKay, V.L., Mai, B., Waters, L., and Breeden, L.L. (2001). Early cell cycle box-mediated transcription of CLN3 and SWI4 contributes to the proper timing of the G(1)-to-S transition in budding yeast. Mol. Cell. Biol. *21*, 4140–4148.

Marini, N.J., and Reed, S.I. (1992). Direct induction of G1-specific transcripts following reactivation of the Cdc28 kinase in the absence of de novo protein synthesis. Genes Dev. *6*, 557–567.

McDonald, W.H., and Yates, J.R., III (2002). Shotgun proteomics and biomarker discovery. Dis. Markers *18*, 99–105.

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

Mendenhall, M.D., and Hodge, A.E. (1998). Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. *62*, 1191–1243.

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

Nevins, J.R. (2001). The Rb/E2F pathway and cancer. Hum. Mol. Genet. *10*, 699–703.

Partridge, J.F., Mikesell, G.E., and Breeden, L.L. (1997). Cell cycle-dependent transcription of CLN1 involves swi4 binding to MCB-like elements. J. Biol. Chem. *272*, 9071–9077.

Polymenis, M., and Schmidt, E.V. (1997). Coupling of cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast. Genes Dev. *11*, 2522–2531.

Reid, R.J., Sunjevaric, I., Keddache, M., Rothstein, R., and Kedacche, M. (2002). Efficient PCR-based gene disruption in Saccharomyces strains using intergenic primers. Yeast *19*, 319–328.

Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. (1989). An essential G1 function for cyclin-like proteins in yeast. Cell *59*, 1127–1133.

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat. Biotechnol. *17*, 1030–1032.

Schwob, E., and Nasmyth, K. (1993). CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genes Dev. *7*, 1160–1175.

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

Simon, I., Barnett, J., Hannett, N., Harbison, C.T., Rinaldi, N.J., Volkert, T.L., Wyrick, J.J., Zeitlinger, J., Gifford, D.K., Jaakkola, T.S., and Young, R.A. (2001). Serial regulation of transcriptional regulators in the yeast cell cycle. Cell *106*, 697–708.

Spellman, P.T., Sherlock, G., Zhang, M.Q., Iyer, V.R., 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.

Stevens, C., and La Thangue, N.B. (2003). E2F and cell cycle control: a double-edged sword. Arch. Biochem. Biophys. *412*, 157–169.

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

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.

Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. Nature *425*, 859–864.

Wijnen, H., and Futcher, B. (1999). Genetic analysis of the shared role of CLN3 and BCK2 at the G(1)-S transition in Saccharomyces cerevisiae. Genetics *153*, 1131–1143.

Wijnen, H., Landman, A., and Futcher, B. (2002). The G(1) cyclin Cln3 promotes cell cycle entry via the transcription factor Swi6. Mol. Cell. Biol. *22*, 4402–4418.

Wittenberg, C., and Flick, K. (2003). Cell cycle regulation during G1 phase in yeast: decisions, decisions, decisions. In G1 Phase Progression, J. Boonstra, ed. (Georgetown, TX: Landes Biosciences), pp. 14–39.

Wolters, D.A., Washburn, M.P., and Yates, J.R., III (2001). An automated multidimensional protein identification technology for shotgun proteomics. Anal. Chem. *73*, 5683–5690.

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.