Dual Regulation by Pairs of Cyclin-Dependent Protein Kinases and Histone Deacetylases Controls G1 Transcription in Budding Yeast

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Abstract

START-dependent transcription in Saccharomyces cerevisiae is regulated by two transcription factors SBF and MBF, whose activity is controlled by the binding of the repressor Whi5. Phosphorylation and removal of Whi5 by the cyclin-dependent kinase (CDK) Cln3-Cdc28 alleviates the Whi5-dependent repression on SBF and MBF, initiating entry into a new cell cycle. This Whi5-SBF/MBF transcriptional circuit is analogous to the regulatory pathway in mammalian cells that features the E2F family of G1 transcription factors and the retinoblastoma tumor suppressor protein (Rb). Here we describe genetic and biochemical evidence for the involvement of another CDK, Pcl-Pho85, in regulating G1 transcription, via phosphorylation and inhibition of Whi5. We show that a strain deleted for both PHO85 and CLN3 has a slow growth phenotype, a G1 delay, and is severely compromised for SBF-dependent reporter gene expression, yet all of these defects are alleviated by deletion of WHI5. Our biochemical and genetic tests suggest Whi5 mediates repression in part through interaction with two histone deacetylases (HDACs), Hos3 and Rpd3. In a manner analogous to cyclin D/CDK4/6, which phosphorylates Rb in mammalian cells disrupting its association with HDACs, phosphorylation by the early G1 CDKs Cln3-Cdc28 and Pcl9-Pho85 inhibits association of Whi5 with the HDACs. Contributions from multiple CDKs may provide the precision and accuracy necessary to activate G1 transcription when both internal and external cues are optimal.

Introduction

Cyclin-dependent protein kinases (CDKs) act as molecular machines that drive cell division, and cell cycle progression is dependent on oscillation between CDK active and inactive states. In S. cerevisiae, the CDK Cdc28 associates with nine different cyclin subunits to promote and coordinate a complex network of events necessary for smooth cell cycle transitions [1]. Irreversible commitment to a new round of cell division occurs toward the end of G1 phase in a process called Start in yeast. The analogous regulatory event is called the restriction point in mammalian cells [2,3]. In yeast, three G1 cyclins, Cln1, Cln2, and Cln3, associate with Cdc28 to initiate events required for progression through Start. Passage through Start catalyzes a defined molecular program that initiates DNA replication, budding, spindle maturation, and chromosome segregation [3].

One key feature of Start in yeast, and G1 progression in other eukaryotic cells, is the induction of a transcriptional program involving over 200 genes, including those encoding the G1 (CLN1, CLN2, PCL1, and PCL2) and B-type cyclins (CLB5 and CLB6) [4,5]. G1/S phase-specific transcription depends on two heterodimeric transcription factors called SBF (Swi4,6 cell cycle box binding factor) and MBF (MluI binding factor). These complexes share a common regulatory subunit, Swi6, which is tethered to DNA via its binding partners, encoded by SWI4 in SBF and MBP1 in MBF [5]. At the well-studied HO locus, binding of the zinc-finger transcription factor Swi5 is followed by recruitment of the Swi/Snf chromatin remodeling complex and the SAGA histone acetyltransferase complex [6–8]. These events set the stage for SBF binding and recruitment of the SRB mediator complex [6]. Importantly, subsequent recruitment of PolII and transcription initiation is dependent on CDK activity [9]. Although any one of the three G1 cyclins is sufficient to drive Start, genetic studies indicate a key role for Cln3-Cdc28 in activating SBF and MBF. At the same time Cln1 and Cln2 are required for the proper
execution of other Start-related events such as budding and DNA synthesis. Cells lacking 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 [10].

Start does not occur until cells have passed a critical cell size threshold, a barrier modulated by nutrient conditions, among other regulatory inputs [11]. A systematic analysis of cell size profiles for the entire set of yeast deletion mutants uncovered many new regulators of Start including Whi5 and implicated it as an inhibitor of G1/S-specific transcription [12,13]. Whi5 occupies specific promoters early in G1 phase when CDK activity is low. However, Cdc28-dependent phosphorylation of both Whi5 and SBF/MBF late in G1 phase results in disengagement from SBF and nuclear export of Whi5 consequently leading to activation of SBF- and MBF-dependent transcription [12,13].

Whi5 is proposed to function in a manner analogous to the well-characterized Rb family proteins in metazoans. E2F, the functional analog of SBF/MBF, regulates G1-specific gene expression required for passage through the restriction point [14]. E2F activity is restricted to late G1 phase because of inhibition by the retinoblastoma protein (Rb). Rb associates with E2F to restrain its activity until late G1, at which point stepwise phosphorylation of Rb by two CDKs, cyclin D-Cdk4/6 and cyclin E-Cdk2, causes the dissociation of Rb from E2F [15]. This process appears to be regulated by a positive feedback loop in which Rb phosphorylation by cyclinE-Cdk2 leads to further dissociation of Rb from promoters and enhancement of G1-transcription. At the molecular level, Rb interacts with both E2F and chromatin remodeling complexes such as histone deacetylases (HDACs) [16–18]. Rb appears to repress transcription through at least three distinct mechanisms: (1) Rb can bind directly to the activation domain of E2F thereby blocking its activity [19]; (2) recruitment of Rb can block the assembly of the pre-initiation complex thus inhibiting the activity of adjacent transcription factors [20] and; (3) Rb can recruit remodelers such as HDAC1 and BRG1 to modify chromatin structure. BRG1 is one of the human Swi/Snf adenine triphosphatases (ATPases) that remodel nucleosomes by utilizing ATP to weaken the interactions between DNA and histones [16,17]. The specific roles of different CDKs in regulating E2F-Rb function, however, remain unclear.

Another yeast CDK Pho85 was originally discovered as a regulator of phosphate metabolism, but has since been shown to play numerous roles in the regulation of cell division and other processes [21–23]. Ten genes encoding Pho85 cyclins (Pcls) have been identified and they appear to dictate substrate and functional specificity of Pho85 [24–26]. Expression of three Pcls, PCL1, PCL2, and PCL9, is restricted to G1 phase of the cell cycle [25]. Specifically, PCL9 expression peaks early in G1, whereas maximal expression of PCL1 and PCL2 is observed at Start and is dependent largely on SBF [27–29]. Although Pho85 is not essential for viability, it is required for cell cycle progression in the absence of the Cdc28 cyclins CLN1 and CLN2 [29], and its absence leads to catastrophic morphogenic changes that culminate in a G2 arrest [30]. Consistent with this observation, inactivation of both Cdc28 and Pho85 CDKs specifically inhibits expression of G1-regulated genes involved in polarized growth [31].

As noted above, transcriptional repression by Rb has been linked to its interaction with histone modification complexes, in particular HDACs. Recent work highlights the importance of post-translational modifications of histone and nucleosome positioning in regulating gene expression [32,33]. Histone acetylation neutralizes the positive charge generated by lysine-rich regions present in the N-terminal tails of histones, thereby disrupting nucleosome structure and increasing promoter accessibility [34]. As a result, many transcription activators have been shown to interact with histone acetyltransferases, whereas transcriptional repressors often associate with HDACs to promote nucleosome formation to occlude transcription factor binding [35,36]. Histone deacetylation in S. cerevisae is mediated by a family of HDACs including Rpd3, Hda1, Hda2, Hos1, Hos2, and Hos3 [37]. Similar to their mammalian counterparts, some yeast HDACs are recruited to promoters by sequence-specific regulatory factors to repress gene expression. For example, the Rpd3 deacetylase complex is recruited to the INO1 promoter by the DNA binding protein Ume6 [35,38–40]. This recruitment results in local histone deacetylation and repression of INO1 gene expression [41]. Hda1 is another example of this type of regulation, and is recruited to its target promoters by the repressor Tup1 [42].

In this study, we provide detailed mechanistic insights into Whi5-dependent regulation of G1-specific transcription and cell cycle progression. Specifically, we identify Whi5, to our knowledge, as the first demonstrated physiological substrate for the G1-specific Pcl9-Pho85 CDK and provide genetic and biochemical evidence supporting a direct role for Pho85 at Start. Furthermore, we show that in a manner similar to Rb in mammalian cells, Whi5-mediated repression involves the HDACs Rpd3 and Hos3. Dual phosphorylation of Whi5 by Cdc28 and Pho85 inhibits Whi5 activity in at least two ways. Both kinases appear to regulate interaction of Whi5 with different HDACs, whereas Cdc28 is also involved in disrupting Whi5 association with SBF and promoting its nuclear export [12,13]. G1-specific CDKs thus are specialized to regulate different aspects of the same critical cell cycle event—inhibition of Whi5—resulting in definitive inactivation of the Whi5 repressor.
Results
A Synthetic Dosage Lethality Screen Identifies Whi5 as a Putative Substrate for the CDK Pho85

Synthetic dosage lethality (SDL) is a genetic assay that is based on the rationale that increasing levels of a protein may have no effect on the growth of an otherwise wild-type (wt) strain but may cause a measurable phenotype—such as lethality—in a mutant strain with reduced activity of an interacting protein [43,44]. Previous studies suggest that SDL can be used effectively to identify novel enzyme targets and a genome-wide SDL screen in cells lacking Pho85 identified known targets of the CDK [23]. In addition to known substrates, several putative Pho85 targets were also identified, including the G1-specific transcription repressor Whi5 [24]. To further explore the role of Pho85 in G1 phase-specific transcription we examined the WHI5-PHO85 SDL interaction in greater detail. As noted previously, Pho85 activity and substrate specificity depends on its interaction with cyclin subunits known as Pcls [25]. To implicate specific Pcl-Pho85 complexes in modulating Whi5 function we examined the effects of WHI5 overexpression in cells lacking different Pcls (Figure 1). Similar to effects observed in cln3Δ and cln1Δ cln2Δ mutants [13], overexpression of WHI5 resulted in growth inhibition of pcl1Δ and pcl9Δ deletion strains and this growth defect was exacerbated in a pcl1Δ pcl9Δ double mutant (Figure 1). Unlike pcl1Δ or pcl9Δ mutants, strains lacking PCL2 or PHO80 cyclins were not adversely affected by increased WHI5 dosage suggesting that the WHI5-PHO85 genetic interaction is dependent on the PCL1,2 cyclin subfamily and more specifically on PCL1 and PCL9 (Figure 1). This observation is consistent with the fact that Pcl1 and Pcl9 (but not Pcl2) are the two G1-specific cyclins that localize to the nucleus [30,45]. The growth phenotype seen in the plating assay was confirmed by measuring growth rates in liquid culture (unpublished data). On the basis of these results, Pcl1/9-Pho85 may contribute to Whi5 regulation in a manner similar to Cln3-Cdc28.

Whi5 Is a Substrate for Pcl9-Pho85 Phosphorylation

The genetic interactions described above suggest Whi5 may be a direct target of Pho85. Evidence supporting this hypothesis is provided by protein microarray assays where Whi5 is phosphorylated in vitro by Pcl1-Pho85 [46]. We characterized the Whi5-Pho85 interaction biochemically by performing in vitro kinase assays using recombinant Pcl-Pho85 CDK complexes and purified Whi5 as substrate (Figure 2A). Incorporation of [32P] into Whi5 was not detected in the absence of CDKs (Figure 2A, lane 4). However, Whi5 phosphorylation was observed in the presence of Pcl1- and Pcl9-Pho85 (Figure 2A, lanes 1,2) and when compared to Cln2-Cdc28 kinase activity, Pho85 and Cdc28 phosphorylated Whi5 at similar levels in vitro (Figure 2A, lanes 1–3).

Previous studies revealed multiple Whi5 slow-migrating isoforms that correlate with its phosphorylation state [12,47]. We examined the effect of various cyclin or CDK mutants on Whi5 mobility (Figure 2B). Because of genetic redundancy of Pcl cyclins [27], we were unable to reproducibly detect changes in Whi5 phosphoforms in cyclin mutant strains. Therefore, a Pho85 mutant was used to

Figure 1. WHI5 overexpression is toxic to strains compromised for Pho85 CDK activity. Isogenic wt (BY263), pho85Δ (BY391), pcl1Δ (BY628), pcl2Δ (BY451), pcl9Δ (BY694), pcl1Δ pcl9Δ (BY760), and pho80Δ (BY490) strains bearing either GAL1-WHI5 (pBA1973) or empty vector control (pEG-H) were spotted in serial 10-fold dilutions on galactose media and incubated for 72 h at 30°C.

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asses the phosphorylation status of Whi5. Consistent with previous findings [12,13], slow migrating Whi5 isoforms present in asynchronous wt extracts (Figure 2B, lane 1) were modestly reduced in cells lacking CLN3 (Figure 2B, lane 7) and completely absent in a cln1Δ cln2Δ double mutant (Figure 2B, lane 6), confirming that Whi5 phosphorylation depends on Cln-Cdc28 kinase complexes. Consistent with our SDL results and in vitro kinase assays, we observed a significant reduction in Whi5 mobility in extracts from a pho85Δ mutant strain (Figure 2B, lane 2). Thus, similar to Cdc28, phosphorylation of Whi5 also depends on Pho85 in vivo.

To determine if Whi5 physically associates with Pho85 in yeast, we first assayed Whi5FLAG immune complexes for kinase activity. A robust autophosphorylation activity was recovered from Whi5FLAG immunoprecipitates derived from wt cell extracts when radiolabeled ATP was added to the immunoprecipitated sample (Figure 2C, lane 2). This activity was partially dependent on both CDC28 and PHO85 (Figure 2C, lanes 3–5). We also confirmed a physical interaction between Whi5 and Pcls using a co-immunoprecipitation assay (Figure 2D). Immunoprecipitation of Whi5MYC from epitope-tagged cyclin extracts revealed a specific
association between Pcl9 and Whi5 (Figure 2D, lane 4). We failed to reproducibly detect a physical interaction between Whi5 and Pcl1 (Figure 2D, lane 2) suggesting that Pcl9-Pho85 is the primary Whi5 CDK. Taken together, the phosphorylation and co-immunoprecipitation assays strongly suggest that, in addition to Cdc28, Pho85 also phosphorylates Whi5. Furthermore, these results identify Whi5 as the first reported substrate for Pcl9-Pho85, one of two Pcls whose activity is restricted to early G1 phase.

Whi5 associates indirectly with G1 phase-regulated promoters through interaction with SBF and MBF. Interactions with these transcription factors and subsequent promoter binding are disrupted by CDK-dependent phosphorylation [12,13]. Because Whi5 appears to be a Pho85 substrate, we assessed the occupancy of SBF promoters by Pcl9. To date, cyclins have not been detected at yeast promoters. Pcl9 is normally an unstable short-lived protein [27]; however, similar to other cyclins, Pcl9 turnover appears to be catalyzed in part by its cognate CDK, Pho85 (Figure 3A) [48]. Therefore, to test Pcl9 promoter localization in a more sensitive genetic background, we performed ChIP (Chromatin immunoprecipitation) experiments in a pho85D strain (Figure 3B). The highest levels of CLN2 promoter DNA were detected in Pcl9NVC: immune complexes 30 min following release from a metaphase-anaphase arrest (Figure 3B). The Pcl9-chromatin association was no longer detectable 45 min after GAL-CDC20 induction indicating that the interaction is short-lived and transient as predicted for a regulator of Start. The association was Whi5-dependent since Pcl9 was not detected at the CLN2 promoter in a strain lacking Whi5 (Figure 3C). The localization of Pcl9 to CLN2, a G1 promoter, is consistent with a direct role for Pcl9-Pho85 in regulating G1 transcription.

Pcl9-Pho85 Regulates Whi5 Function via Phosphorylation

As mentioned above, cln3D mutants arrest in G1 phase as large unbudded cells in response to increased WHI5 dosage, indicating that Whi5 is a dose-dependent regulator of Start. Therefore, if Pho85 and Cdc28 function analogously to inhibit Whi5 activity, we predict that elevated Pho85 kinase activity would antagonize the toxic effects of WHI5 overexpression and suppress the growth defects observed in a cln3D mutant. To test this prediction, high copy plasmids expressing PCL1, PCL2, PCL9, or PHO80 were introduced into a cln3D strain expressing WHI5 from a conditional MET25 promoter (Figure 4A). Plasmid-based expression of Pcls and Whi5 was confirmed by immunoblotting (Figure S1). Induction of WHI5 expression in a cln3D mutant resulted in cell death whereas overexpression of PCL1 or PCL9 partially suppressed this toxicity and restored growth (Figure 4A). Consistent with results from SDL analyses (Figure 1), this suppression was specific to PCL1 and PCL9 since neither PCL2 nor PHO80 were able to function effectively in the assay (Figure 4A). Furthermore, PCL1/9-mediated suppression was dependent on phosphorylation since growth of a cln3D mutant expressing a nonphosphorylatable form of WHI5 (Whi512A) [13] could not be restored (Figure 4A). These genetic results corroborate the biochemical evidence that Pcl-Pho85 regulates Whi5 activity through phosphorylation.

Given its effect on WHI5 overexpression, we next examined PCL effects on other CLN3-associated phenotypes. CLN3 is required to activate G1-specific transcription once cells have achieved a critical size [49–51]. A cln3D mutant exhibits a large cell size phenotype because of its inability to inhibit Whi5 and activate Start-specific transcription [12,13]. Ectopic expression of PCL1 or PCL9 reduced cln3D cell size to an intermediate level between that of wt and cln3D cells (Figure 4B). Conversely, deletion of PCL9, PCL1, and the partially redundant cyclin PCL2 resulted in a cell size increase (Figure 4C). These results suggest that Pcl-Pho85 and Cln3-Cdc28 share a common role in cell cycle progression to regulate Whi5 activity and promote passage through Start.

CDC28 and PHO85 Function in Parallel Pathways to Regulate Whi5 Function

To determine if Pcl-Pho85 and Cln3-Cdc28 might function in parallel to regulate Start, we first tested whether pcl1D cln3D or pcl1D pcl3D cln3D strains showed any synthetic growth defects. As expected, no growth defects were observed, probably because of the redundant effects of other Pcls [27]. Unlike the Cdc28 cyclins, which shows distinct cell cycle expression patterns, most Pcls are expressed at all cell cycle stages [25]. We then examined the phenotype of a pho85D cln3D double mutant. Cells lacking cln3D are larger than wt cells but do not display overt defects in growth rate while pho85D mutants are slow growing (Figure 5A). However, pho85Dcln3D double mutants exhibited a more pronounced growth defect compared to single mutants and analysis of DNA content revealed that the pho85D cln3D double mutant cells accumulated in G1 phase with predominantly unreplicated DNA (Figure 5A). Importantly, deleting WHI5 overcame both the cell cycle progression and growth defects observed in the absence of both CLN3 and PHO85. Notably, a pho85D cln3D whi5D triple mutant exhibited a growth rate similar to a cln3D single mutant indicating that Pcl-Pho85 and Cln3-Cdc28 function in separate yet converging pathways to regulate Whi5 function and, by extension, G1 cell cycle progression (Figure 3A). These observations also hold true under liquid growth conditions as shown. Whi5-dependent suppression appears to be specific to the pho85D cln3D phenotype because WHI5 deletion was unable to rescue 53 additional synthetic lethal interactions involving PHO85 (Table S1; D.Q. Huang and B.J. Andrews, unpublished data).

Given that Whi5 represses SBF- and MBF-specific transcription, we asked whether PHO85 affects SBF-driven reporter gene expression. A reporter gene consisting of tandem SCB consensus element repeats fused upstream of the HIS3 coding region was constructed and integrated into wt, cln3D, and pho85D strains. Previous work has shown that this reporter provides a highly specific read-out for SBF-dependent transcription [13,52]. Growth on medium lacking histidine supplemented with 3-aminotriazole (3-AT) was used to assess SBF transcriptional activity (Figure 5B). Even though cells lacking PHO85 were moderately sensitive to higher concentration (5 mM) of 3-AT (unpublished data), both cln3D and pho85D mutants showed no growth in media containing 30 mM 3-AT indicating that SBF transcription is impaired in these mutants, whereas growth of wt cells was unaffected [13]. Furthermore, defects in SBF-driven gene expression were more pronounced in the pho85D cln3D double mutant (at 10 mM 3-AT, Figure 5B). Consistent with the genetic interactions described above (Figure 5A), SBF-dependent reporter activity was restored in pho85D cln3D mutants when WHI5 was deleted (Figure 5B). However, WHI5 deletion only partially rescued the growth defect in pho85D cln3D cells at 30 mM of 3-AT (Figure 5B). The Whi5-independent 3-AT sensitivity of pho85D cells may be due to unregulated Gcn4 in the absence of PHO85, since Gcn4 is induced by 3-AT and Pho85 has been shown to regulate Gcn4 stability [53,54]. Nonetheless, these data suggest that, like Cln3-Cdc28, Pcl-Pho85 modulates SBF activity through Whi5.

We next interrogated the effects of CDK activity on Whi5-mediated transcriptional repression (Figure 6). A construct expressing a LexA DNA binding domain fused to WHI5 was introduced into a strain harboring a LexA reporter gene containing LexA binding sites in its promoter (Figure 6). Consistent with its role as a negative regulator of G1-specific transcription, a ~10-fold reduction in β-galactosidase activity was observed in cells
Figure 3. Pcl9 localizes to G1-specific promoters in a cell cycle-dependent manner. (A) Pho85 regulates Pcl9 protein stability. Wt (BY263) and pho85Δ strains (BY391) harboring a GAL1-PCL9HA plasmid (pBA2112) were grown to exponential phase in galactose media (lane 1). PCL9 expression was repressed by addition of glucose to final concentration of 2% and cells were harvested 10 (lane 2), 30 (lane 3), and 90 (lane 4) min after addition of glucose. Pcl9 abundance was assessed by immunoblotting using 12CA5 anti-HA antibodies. (B) Pcl9 localizes to SBF-dependent promoters. An exponentially growing GAL1-CDC20 pho85Δ PCL9MYC strain (BY4148, lane 1) was arrested at M/G1 phase in glucose-containing medium (lane 2). Cultures were harvested 15 (lane 3), 30 (lane 4), 45 (lane 5), and 60 (lane 6) min after release from CDC20-induced arrest in galactose medium. Cell cycle progression was monitored by FACS analysis. Anti-MYC and anti-Swi6 ChIPs from the indicated strains were analyzed for CLN2 promoter sequences by quantitative RT-PCR. (C) In a strain lacking Whi5, GAL1-CDC20 pho85Δ whi5Δ PCL9MYC, Pcl9 no longer localizes to the CLN2 promoter. Anti-Swi4 ChIPs are shown as a positive control.

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expressing the LexA-Whi5 fusion protein compared to a vector control (Figure 6). Overexpression of PCL9, CLN3, or CLN2 restored LacZ expression to intermediate levels indicating that activation of either CDC28 or PHO85 was capable of antagonizing Whi5 function in this assay (Figure 6). Consistent with suppression of WHI5-mediated growth defects (Figure 4), inhibition of Whi5 activity was dependent on phosphorylation since LacZ expression could not be restored in cells harboring an unphosphorylatable LexA-Whi512A fusion protein (Figure 6).

Pho85 Does Not Regulate Whi5 Localization or Its Interactions with G1-Specific Transcription Complexes

Cln2-Cdc28 activity was previously shown to disrupt recombinant Whi5-SBF complexes in vitro [13], but Cln3-Cdc28 and Pho85 kinases had not been assessed for this activity. A preassembled recombinant Whi5-Swi4FLAG-Swi6 complex bound to anti-FLAG resin was incubated with purified kinases in the presence of radiolabeled ATP and separated into soluble (Figure 7B, labeled “S”) and bound fractions (Figure 7B, labeled...
Figure 5. **PHO85 regulates G1 transcription via WHI5.** (A) The G1 delay phenotype associated with a cln3Δ pho85Δ strain is dependent on WHI5. Wt (BY263), cln3Δ (BY653), pho85Δ (BY391), cln3Δ pho85Δ (BY4291), and cln3Δ pho85Δ whi5Δ (BY4292) strains were spotted in serial 10-fold dilutions on rich media (YPED) and incubated for 24 h at 30°C. DNA content of exponentially growing cultures was determined by FACS analysis. Liquid growth assays were also performed for these strains and growth rate is reported relative to wt as shown in the bar graph. Graphical representations of growth rates are shown above the bar graph as line plots, where the upper red line represents the growth of WT and the black line shows the growth of each mutant. (B) A cln3Δ pho85Δ strain exhibits defects in SCB-driven gene expression. Wt (BY4302), cln3Δ (BY4303), pho85Δ (BY4304), cln3Δ pho85Δ (BY4305), cln3Δ pho85Δ whi5Δ (BY4306), pho85Δ whi5Δ (BY4308), and cln3Δ whi5Δ (BY4307) strains harboring an integrated SCB-HIS3 reporter were spotted in serial 10-fold dilutions on histidine-containing medium or media lacking histidine and supplemented with 10 or 30 mM 3-AT. Plates were incubated at 30°C for 48 h. We note that the synthetic growth defect of a cln3 pho85 mutant is most pronounced on rich medium (A), and is not as evident when strains are grown on minimal medium.

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Mechanism for Whi5-Mediated Transcriptional Repression by Pho85

We next explored what additional mechanism might explain Pcl- and Cln3-mediated regulation of Whi5 activity. Functional conservation clearly extends to Whi5 and its metazoan analogue Rb [14]. Since Rb represses transcription, in part, through recruitment of HDACs, we used a batch affinity chromatography assay to test for physical interactions between a Whi5-GST ligand and tandem affinity tagged HDACs (Figure 8A). Specific interactions between Whi5 and Hos3, Rpd3, and, to a lesser extent, Hos1 were identified (Figure 8A, lanes 1, 5, 13) suggesting that, like Rb, Whi5-dependent transcriptional repression involves recruitment of HDACs. This observation is consistent with previous work that detected Rpd3 at the PCL1 promoter using a ChIP assay [55]. Furthermore, Hos3 and Rpd3 were required for WHI5 dose-dependent effects on cell size. Like wt cells, strains lacking either Hos3 (Figure 8B, panel 1) or Rpd3 (Figure 8B, panel 2) also exhibited a dose-dependent increase in cell size in response to WHI5 overexpression. However, additional cell size effects were not observed in strains lacking both HDACs, suggesting that Hos3 and Rpd3 regulate Whi5 function synergistically (Figure 8B, panel 3).

If HDACs are required for Whi5 function, then strains lacking HDAC function should be resistant to toxic effects associated with WHI5 overexpression. Consistent with this prediction, the growth defect caused by WHI5 overproduction in a cln3Δ was alleviated by the deletion of Hos3 and Rpd3 [Figure 9A]. Deletion of Hos3 alone rescued WHI5 toxicity in a pho85Δ strain while a cln3Δ mutant required deletion of both Hos3 and Rpd3 in order to tolerate increased dosage of WHI5 (Figure 9A).

Given that Whi5 appears to be acting through HDACs, we predicted that deletion of Hos3 and Rpd3 should phenocopy those genetic interactions seen in whi5Δ mutants. We first tested various HDAC deletion strains for suppression of the slow growth phenotype of a pho85Δcln3Δ mutant. As for WHI5, deletion of Hos3 and Rpd3 partially suppressed the growth defect seen in the pho85Δcln3Δ double mutant strain (Figure 9B). Suppression was specific to Hos3 and Rpd3 because deletion of other HDACs showed no suppression, and the growth rate of the pho85Δcln3Δ-hos3Δ strain was not improved by subsequent deletion of Rpd3 and vice versa (Figure 9B).

We next asked if deletion of HDACs might overcome the Start arrest seen in cells lacking both CLN3 and Bck2, another regulator of G1 transcription that functions in parallel with CLN3 [56]. A cln3Δbck2Δhos3Δ triple mutant grows as vigorously as wt, placing WHI5 downstream of both upstream activators of G1 transcription [13]. Interestingly, deletion of Rpd3 partially restored growth in the cln3Δbck2Δ strain providing further evidence for an HDAC requirement in Whi5-mediated transcriptional repression (Figure 9C). Neither subsequent deletion of Hos3 nor deletion of other HDACs affected growth appreciably (Figure 9C). We also employed the SCB-HIS3 assays used above to explore SBF-driven reporter gene expression in the HDAC mutants (Figure 10). As expected, deletion of Rpd3 rescued the growth defects of cln3Δ SSB-HIS3 cells in the presence of both 10 mM and 30 mM of 3-AT, whereas Hos3 gene knockout had a marginal but additive effect. In contrast, the growth of pho85Δcells was slightly rescued by deletion of Hos3 but not Rpd3 providing further evidence for Pho85 acting specifically through Hos3. Because of difficulties in detecting HDACs at promoters, we were unable to confirm these observations in vivo.

We also performed co-immunoprecipitation assays using affinity tagged Rpd3 and Hos3 strains and observed an obvious decrease in Rpd3 and Hos3 in Whi5 precipitates from strains harboring increased levels of Pcl9, Cln2, or Cln3 cyclins (Figure 11A and 11B). Together, our genetic and biochemical results suggest that Pho85 may preferentially influence Whi5-Hos3 activity, whereas Cln3-Cdc28 is required for inhibition of both Rpd3 and Hos3.

Figure 6. Whi5-mediated transcriptional repression is antagonized by PHO85 and CDC28. A reporter gene consisting of eight LexA binding sites flanked by the GAL1 promoter and the LacZ coding sequence was constructed (pBA1976). β-galactosidase activity (upper histogram) was measured in a wt strain (BY263) bearing the LacZ reporter along with one of the following: a vector control (pBA230v); a LexA expressing plasmid (pLexA; pBA1977); or a construct expressing a LexA-Whi5 fusion protein (pLexA-WHI5; pBA1978). β-galactosidase activities were also assayed (lower histogram) in a wt strain harboring the LacZ reporter construct alone (vector control; pBA1976) or overexpressing the G1 cyclins, PCL9 (pBA1974), CLN2 (pBA2247), or CLN3 (pBA2248) in the presence of LexA-Whi5 (pBA1978) or LexA-Whi5Δ (pBA1979) fusion proteins.

“B”), Equivalent amounts of kinase were approximated on the basis of in vitro kinase activity [Figure 7A, and Materials and Methods]. As expected, Cln2-Cdc28 phosphorylation caused most of the SBF-bound Whi5 to be released into the soluble fraction (Figure 7B, lanes 3 and 4). In contrast, we failed to observe dissociation of Whi5 from SBF in the presence of Cln3- or Pcl9-CDK complexes (Figure 7B, lanes 5–10). In addition to negatively regulating the interaction of Whi5 with SBF, Cdc28 also controls its localization [13]. Unlike Cln-Cdc28 phosphorylation, which promotes Whi5 export from the nucleus, deletion of PHO85 did not dramatically affect the subcellular localization of Whi5 (Figure 7C). Together, these results suggest that Pho85 must regulate Whi5 function through alternate mechanisms.
Figure 7. Pho85 does not affect known Whi5 regulatory mechanisms. (A) Determination of relative Cdc28 and Pho85 kinase activity. In vitro kinase assays using varying amounts of recombinant Cln2-Cdc28, Cln3-Cdc28, and Pcl9-Pho85 in the absence (lane 1-3) or presence of purified Whi5 (lanes 5-8) were conducted and the degree of Whi5 phosphorylation was determined by SDS-PAGE and autoradiography. Purified Whi5 and γ-32P-ATP were incubated in the absence of kinase in lane 8, and lane 4 is empty. A 3 μM final concentration of Cln3-Cdc28 and Pcl9-Pho85 and a 60 nM final concentration of Cln2-Cdc28 give similar amounts of 32P-incorporation in Whi5, although phosphorylation by Cln2-Cdc28 caused Whi5 to migrate more slowly than Whi5 phosphorylated by Cln3-Cdc28 or Pcl9-Pho85. The concentration of kinase used in (B) was based on these experiments. (B) Cln3-Cdc28 and Pcl9-Pho85 do not influence Whi5-SBF complex stability. A preassembled recombinant Whi5-Swi4FLAG-Swi6 complex bound to anti-FLAG resin was incubated with Cln2-Cdc28, Cln3-Cdc28, Pcl9-Pho85, or both Cln3-Cdc28 and Pcl9-Pho85 in the presence of radiolabeled ATP. After washing, proteins in the bound and supernatant fractions were identified by autoradiography. (C) Subcellular localization of Whi5 in cdk mutant strains. Wt (BY263), pho85Δ (BY391), and cdc28-4 strains (BY465) expressing WHI5GFP from a methionine-repressible promoter (pBA1981) were examined for Whi5GFP fluorescence. Representative fields are shown.

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Figure 8. Whi5 function is dependent on HDAC activity. (A) Whi5 associates with Hos3 and Rpd3. Lysates prepared from the indicated epitope-tagged HDAC strains (BY4309–4315) harboring a vector control (pEG-H) or construct expressing WHI5GST (pBA1973) were incubated with glutathione sepharose beads. Whi5GST-HDAC interactions were detected by immunoblot using α-GST and α-PAP antibodies. (B) Hos3 and Rpd3 modulate Whi5 cell size effects. A plasmid expressing WHI5 (pBA1980) or vector control (pBA230v) were introduced into wt (BY263), hos3Δ (BY4293), rpd3Δ (BY4294), and hos3Δ rpd3Δ (BY4295) strains, and cell size distributions were measured. Each panel corresponds to a specific mutant and wt distributions are superimposed in each panel. The median cell volume based on three replicates was: 42.06 fl ± 1.09 (wt + vector control, blue); 73.12 fl ± 1.16 (wt + WHI5, black); 30.57 fl ± 1.23 (hos3Δ + vector control, panel 1, red); 71.35 fl ± 1.59 (hos3Δ + WHI5, panel 1, green); 51.20 fl ± 1.73 (rpd3Δ + vector control, panel 2, red); 69.75 fl ± 2.79 (rpd3Δ + WHI5, panel 2, green); 45.62 fl ± 1.22 (hos3Δ rpd3Δ + vector control; panel 3, red); 50.26 fl ± 1.14 (hos3Δ rpd3Δ + WHI5, panel 3, green).

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Figure 9. \textit{WHI5} toxicity is dependent on \textit{HOS3} and \textit{RPD3}. (A) $\text{cln3}^{\Delta}$ (BY4290), $\text{cln3}^{\Delta} \text{rpd3}^{\Delta}$ (BY4297), $\text{cln3}^{\Delta} \text{hos3}^{\Delta}$ (BY4296), and $\text{cln3}^{\Delta} \text{rpd3}^{\Delta} \text{hos3}^{\Delta}$ (BY4298) strains harboring a methionine-repressible \textit{WHI5} construct (pBA1975) or vector control (pBA228v) were spotted in serial 10-fold dilutions on medium lacking methionine. In a similar experiment, $\text{pho85}^{\Delta}$ (BY391), $\text{pho85}^{\Delta} \text{rpd3}^{\Delta}$ (BY4300), $\text{pho85}^{\Delta} \text{hos3}^{\Delta}$ (BY4299), and $\text{pho85}^{\Delta} \text{rpd3}^{\Delta} \text{hos3}^{\Delta}$ (BY4301) strains bearing a galactose-inducible \textit{WHI5} plasmid (pBA1973) or appropriate vector control (pEG-H) were spotted in serial 10-fold dilutions on galactose-containing medium. Plates were incubated at 30°C for 48 h. (B) Deletion of \textit{HOS3} partially restores growth of a $\text{cln3}^{\Delta} \text{pho85}^{\Delta}$ strain. The indicated strains (BY263; BY4291, BY4292, BY4455–4461) were spotted in serial 10-fold dilutions on rich medium (YPED) and incubated at 30°C for 48 h. (B) Deletion of \textit{HOS3} partially restores growth of a $\text{cln3}^{\Delta} \text{pho85}^{\Delta}$ strain. The indicated strains (BY263; BY4291, BY4292, BY4455–4461) were spotted in serial 10-fold dilutions on rich medium (YPED) and incubated at 30°C for 48 h. (C) Deletion of \textit{RPD3} and \textit{HOS3} partially restore viability of a $\text{cln3}^{\Delta} \text{bck2}^{\Delta}$ strain. The indicated strains (BY4741; BY2948, BY4462–4468) were spotted in serial 10-fold dilutions on glucose-containing medium (YPED) to repress \textit{CLN3} expression. Strains were also spotted on medium containing galactose as a control. Plates were incubated at 30°C for 72 h.

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Discussion

Whi5 is a critical cell cycle regulator that links CDK activity in G1 phase to the broad transcriptional program that accompanies commitment to cell division. We provide substantial evidence that the multifunctional Pho85 CDK is an important regulator of Whi5 activity and G1 phase-specific transcription including: (1) Whi5 is phosphorylated and antagonized by Pho85 and is the first reported substrate for the G1-specific CDK complex, Pcl9-Pho85; (2) the activity of an SBF-dependent promoter is influenced by PHO85; (3) the Pcl9 cyclin binds to SBF-regulated promoters; (4) the repressor function of Whi5 is mediated through the HDACs Hos3 and Rpd3; and (5) HDAC-Whi5 association is regulated by G1-specific forms of both the Pho85 and Cdc28 CDKs. We therefore conclude that timely and efficient release from Whi5 inhibition and subsequent G1/S cell cycle progression requires the concerted activity of both Cdc28 and Pho85.

Several lines of evidence point to common roles for Pho85 and Cdc28. For example, a burst of both G1-specific Cdc28 and Pho85 activity is both necessary and sufficient to drive these events since neither SBF binding to Whi5 nor nuclear localization of Whi5 was adversely affected in a pho85Δ mutant (Figure 7). Also, we are able to detect binding of SBF in vivo to CLN2 promoters when PHO85 is deleted (Figure 3C). However, both purified Chin3-Cdc28 and Pcl9-Pho85 failed to affect Whi5-SBF stability in vitro, while complex disruption was effectively achieved in the presence of Chin3-Cdc28 and Pcl9-Pho85 may have a more pronounced effect on the Whi5-SBF complex in vivo.

Figure 10. Repression of gene expression by Whi5 is dependent on HOS3 and RPD3. The growth defects of cln3Δ and pho85Δ strains can be rescued by removing RPD3 and HOS3 in SCB-driven gene expression. Wt (BY4302), cln3Δ (BY4303), pho85Δ (BY4304), cln3Δ rpd3Δ (BY4297), cln3Δ hos3Δ (BY4296), cln3Δ rpd3Δ hos3Δ (BY4298), pho85Δ rpd3Δ (BY4300), pho85Δ hos3Δ (BY4299), and pho85Δ rpd3Δ hos3Δ (BY4301) strains harboring an integrated SCB-HIS3 reporter were spotted in serial 10-fold dilutions on histidine-containing medium or media lacking histidine and supplemented with 10 or 30 mM 3-AT. Plates were incubated at 30°C for 48 h.

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strain bearing and Hos3. Experiments were conducted as described in (A) but using a PAP antibodies, (B) Pho85 and Cdc28 activity inhibits interaction of Whi5

Lysates were incubated with glutathione sepharose beads. Whi5GST-expression was confirmed by immunoblot using anti-FLAG antibodies. doi:10.1371/journal.pbio.1000188.g011

Rpd3TAP interactions were detected by immunoblot using control (pMT3164) were introduced into a strain harboring PCL9FLAG, CLN2FLAG, or a vector plasmid (pBA2248), or a vector (pBA1973). Cyclin CLN2FLAG, or a vector (pBA1973).

Figure 11. CDK activity antagonizes Whi5-HDAC interactions. (A) Pho85 and Cdc28 activity inhibits interaction between Whi5 and Rpd3. Rpd3TAP at the chromosomal locus (BY4315) and a WHI5GST plasmid (pBA1973). Cyclin expression was confirmed by immunoblot using anti-FLAG antibodies. Lysates were incubated with glutathione sepharose beads. Whi5GST. Rpd3TAP interactions were detected by immunoblot using α-GST and α-PAP antibodies. (B) Pho85 and Cdc28 activity inhibits interaction of Whi5 and Hos3. Experiments were conducted as described in (A) but using a strain bearing Hos3TAP at the chromosomal locus. doi:10.1371/journal.pbio.1000188.g011

Alternatively, Cln3- and Pcl9-CDKs may act primarily as agonists of HDAC interactions while physical interactions with SBF and nuclear export are optimally mediated by the late G1 CDKs, Cln1- and Cln2-Cdc28. Indeed, recent work reveals activation of CLN2 expression while Whi5 remains bound to the promoter (H. Wang, L.B. Carey, Y. Cai, H. Wijnen, and B. Futcher, personal communication). Such a mechanism may serve to sharpen the onset, as opposed to the timing, of G1/S gene expression thus ensuring a sustained transcriptional burst and irreversible commitment to cell division [13]. Consistent with this idea, recent analysis of cyclin gene expression using a single cell assay affirms that positive feedback involving the Cln1 and Cln2 cyclins induces the G1/S regulon, and that this regulatory feedback is important for maintaining coherence of gene expression at Start [63].

SBF promoter recruitment depends on a series of well-organized chromatin remodeling events [7,36]. SBF, in turn, regulates the recruitment of the general transcription machinery via a two-step process beginning with the mediator complex followed by CDK-dependent recruitment of RNA PolIII, TFIIH, and TFIIH [9]. Previous studies suggested that this CDK requirement stems from Whi5, which in its unphosphorylated state, remains bound to SBF and occludes the basal transcription machinery from binding specific promoters [13]. We have extended this model to include a role for HDAC activity. We predict that Hos3 and Rpd3 contribute to Whi5 repression by preventing holoenzyme access to chromatin. During states of high CDK activity, Cdc28 and Pho85 abrogate Whi5-HDAC and Whi5-SBF interactions and initiate transcription. Consistent with our model, Pcl9 and Cln3 cyclins localize to G1 promoters and Whi5 remains associated with G1-specific promoters in the absence of HDAC-promoter interactions (Figure 3; H. Wang, L.B. Carey, Y. Cai, H. Wijnen and B. Futcher, personal communication). However, Whi5 may also repress transcription by additional mechanisms since its activity is partially retained in hos3Δ rpd3Δ mutants (Figure 9).

Rpd3 is a well-characterized HDAC that accomplishes most of its functions as part of a large protein complex [37]. The Rpd3-Sin3 deacetylase complex has long been implicated as a cell cycle regulator required for silencing H0 gene expression to prevent mating type switching in newly budded cells [64,65]. Our observations that Whi5 associates with Rpd3 and our genetic data linking G1 Cdk5s, Whi5, and Rpd3 reveal a more general role for Rpd3 in G1/S-phase specific transcription. These data are consistent with observations from Butler and colleagues that the Rpd3 protein can be detected at the CLN2 promoter and that the amount of Rpd3 at the promoter is decreased when CLN3 is induced (H. Wang, L.B. Carey, Y. Cai, H. Wijnen and B. Futcher, personal communication). The Rpd3-Sin3 HDAC has also been connected to G1 transcription factors through the interaction of Sin3 with Stb1, a Swi6-binding protein [66–68]. Both Stb1 and Sin3 are required for repression of G1 transcription early in G1 phase [68]. Unlike Rpd3, Hos3 is largely uncharacterized, although a recent study suggests a role for Hos3 in yeasts upon exposure to oxidative radicals [69]. We have uncovered an additional role for Hos3 in Whi5-mediated transcriptional repression.

A question that arises from our observations is what advantage does combinatorial kinase regulation impart on specific biological processes such as G1/S cell cycle progression? Contributions from multiple CDKs may provide the precision and accuracy necessary for rapid definitive decisions that irreversibly affect cellular fate. Indeed, distributive multisite phosphorylation mechanisms exhibit ultrasensitivity with respect to kinase concentration, thereby creating a “switch-like” behavior in biological circuits [70]. Since cell cycle transitions typically display switch-like attributes, multisite phosphorylation by various kinase combinations may prove to be a rule rather than the exception amongst CDK targets, including key cell cycle regulators such as Whi5. In fact, a recent...
computational analysis showed enrichment of multiple closely spaced consensus sites for Cdc28 substrates in yeast, a pattern that proved predictive of likely CDK targets [71].

Although kinase combinations are likely necessary for cell cycle regulation, the contribution of each individual kinase may vary depending on specific signals and environmental stimuli. In certain environments, Pcl-Pho85 may have more dramatic, condition-specific effects on Whi5 function than Cdc28 analogous, perhaps, to the regulation of Rb that is required for quiescence and prevention of apoptosis [72,73]. Previous studies indicate that Whi5 localizes to nuclei in stationary phase cells suggesting that Whi5 may also play a role in G0 [13]. Interestingly, Pho85 is required for survival in starvation conditions and plays an important role during stationary phase [74–76]. Furthermore, CDK5, the mammalian Pho85 homolog, induces apoptosis in neuronal cells via Rb phosphorylation [77]. Whether Whi5 activity is more prominently affected by Pcl-Pho85 in response to stationary or stress conditions requires additional investigation.

Similarities between metazoan and yeast cell cycle regulation are increasingly evident as we continue to characterize Whi5 function. For example, similar to proposed Pcl9/Cln3 “early” phase regulation (Figure 12), cyclinD-CDK4/6 phosphorylates Rb to promote HDAC dissociation and E2F transcriptional activation. E2F activation then leads to cyclin E expression, which, similar to Cln1/2 “late” phase regulation (Figure 12), may establish a positive feedback loop whereby cyclinE-CDK2 activity disrupts Rb-promoter interactions and stimulates G1-transcription further [15]. Despite these similarities, the importance of multiple regulatory components in both yeast and mammalian systems remains poorly understood and may be most fruitfully dissected using the yeast model.

Materials and Methods

Yeast Strains, Growth Conditions, and Plasmids

The S. cerevisiae strains used are listed in Table 1. All gene disruptions and integrations were achieved by homologous recombination at their chromosomal loci by standard PCR-based methods and confirmed by PCR with flanking primers [78]. Standard methods and media were used for yeast growth and transformation. Two percent of galactose in the media was used to induce the expression of genes under the GAL1 promoter. Synthetic minimal medium with appropriate amino acid supplements was used for cells containing plasmids. Appropriate amounts of 3-AT were added to SD-HIS plates to assess the expression of HIS3 reporter genes. 10-fold serial dilutions (5–10^7) of yeast cells were spotted onto plates with appropriate nutrition conditions to assess growth. Plasmids used in this study are listed in Table 2. In most cases, a DNA insert was amplified by PCR and inserted into a linearized vector by homologous recombination in yeast. Details of construction will be provided upon request.
| Strain       | Genotype                          | Source or Reference |
|-------------|----------------------------------|---------------------|
| BY186       | BY263 MATa swi4ΔHIS3             |                     |
| BY263       | MATa trp1 leu2 his3 ura3 lys2 ade2 |                     |
| BY391       | BY263 MATa pho85ΔLEU2            |                     |
| BY451       | BY263 MATa pcl2ΔLYS2             |                     |
| BY462       | MATa leu2 his3 ura3 css28-13     | M. Tyers            |
| BY465       | MATa leu2 his3 ura3 css28-4      | M. Tyers            |
| BY490       | BY263 MATa pho80ΔHIS3            |                     |
| BY628       | BY263 MATa pcl1ΔLEU2             |                     |
| BY653       | BY263 MATa clin3ΔURA3            |                     |
| BY694       | BY263 MATa pcl9ΔHIS3             |                     |
| BY760       | BY263 MATa pcl1ΔLEU2 pcl9ΔHIS3   |                     |
| BY764       | BY263 MATa pcl1ΔLEU2 pcl2ΔLYS2 pcl9ΔHIS3 |                     |
| BY867       | BY263 MATa pho85ΔTRP1            |                     |
| BY1446      | BY263 MATs clin3ΔURA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY1502      | Y2454 MATs pho85ΔLEU2            |                     |
| BY2507      | BY4741 MATa WHI5mycΔ::KAN6        |                     |
| BY2948      | BY4741 MATa clin3ΔHPFH bck2ΔNAT6 pGAL-CLN3 URA3 | This study      |
| BY4148      | BY4741 MATa GALpr-HA-CDC20::KAN6 pho85ΔNAT6 pcl9Δmyc | This study      |
| BY4151      | BY4741 MATa GALpr-HA-CDC20::KAN6 |                     |
| BY4152      | BY4741 MATa WHI5mycΔ::KAN6 pho85ΔNAT6 | This study      |
| BY4153      | BY4741 MATa WHI5mycΔ::KAN6 css28-4 | This study      |
| BY4154      | BY4741 MATa WHI5mycΔ::KAN6 css28-4 pho85ΔNAT6 | This study      |
| BY4242      | BY4741 MATa GALpr-HA-CDC20::KAN6 clin1ΔNAT6 clin2ΔHPFH | This study      |
| BY4269      | BY4741 MATs GALpr-HA-CDC20::KAN6 clin3ΔURA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4270      | BY4741 MATa GALpr-HA-CDC20::KAN6 clin3ΔURA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4273      | BY4741 MATa GALpr-HA-CDC20::KAN6 clin3ΔURA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4274      | BY4741 MATa GALpr-HA-CDC20::KAN6 pho85ΔLEU2 pho85ΔNAT6 | This study      |
| BY4288      | BY4741 MATa WHI5mycΔ::KAN6 clin3ΔLEU2 | This study      |
| BY4289      | BY4741 MATa WHI5mycΔ::KAN6 clin1ΔNAT6 clin2ΔHPFH | This study      |
| BY4290      | BY263 MATa clin3ΔTRP1            |                     |
| BY4292      | BY263 MATs clin3ΔURA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4293      | BY263 MATa hos3ΔKAN6             |                     |
| BY4294      | BY263 MATa rpd3ΔNAT6             |                     |
| BY4295      | BY263 MATa hos3ΔKAN6 rpd3ΔNAT6   |                     |
| BY4296      | BY263 MATa clin3ΔTRP1 hos3ΔKAN6  |                     |
| BY4297      | BY263 MATa clin3ΔTRP1 rpd3ΔNAT6  |                     |
| BY4298      | BY263 MATa clin3ΔTRP1 hos3ΔKAN6 rpd3ΔNAT6 | This study      |
| BY4299      | BY263 MATa pho85ΔLEU2 hos3ΔKAN6  |                     |
| BY4300      | BY263 MATa pho85ΔLEU2 rpd3ΔNAT6  |                     |
| BY4301      | BY263 MATa pho85ΔLEU2 hos3ΔKAN6 rpd3ΔNAT6 | This study      |
| BY4302      | BY4741 MATa hoΔ::SCB::HIS3::URA3 |                     |
| BY4303      | BY4741 MATa hoΔ::SCB::HIS3::URA3 clin3ΔNAT6 | This study      |
| BY4304      | BY4741 MATa hoΔ::SCB::HIS3::URA3 pho85ΔLEU2 | This study      |
| BY4305      | BY4741 MATa hoΔ::SCB::HIS3::URA3 clin3ΔNAT6 pho85ΔLEU2 | This study      |
| BY4306      | BY4741 MATa hoΔ::SCB::HIS3::URA3 clin3ΔNAT6 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4307      | BY4741 MATa hoΔ::SCB::HIS3::URA3 clin3ΔNAT6 whi5ΔKAN6 | This study      |
| BY4308      | BY4741 MATa hoΔ::SCB::HIS3::URA3 pho85ΔLEU2 whi5ΔKAN6 | This study      |
| BY4309      | BY4741 MATa HOS1TA::HIS3          |                     |
| BY4310      | BY4741 MATa HOS2TA::HIS3          |                     |
| BY4311      | BY4741 MATa HOS3TA::HIS3          |                     |

Table 1. Yeast strains.
Table 1. Cont.

| Strain | Genotype | Source or Reference |
|--------|----------|---------------------|
| BY4312 | BY4741 MATa HDA1TAP-HIS3 | This study |
| BY4313 | BY4741 MATa HDA2TAP-HIS3 | This study |
| BY4314 | BY4741 MATa HDA3TAP-HIS3 | This study |
| BY4315 | BY4741 MATa RPD3TAP-HIS3 | This study |
| BY4454 | BY263 MATa whi5ΔΔANΔ | This study |
| BY4455 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 hos1ΔHIS5 | This study |
| BY4456 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 hos2ΔHIS5 | This study |
| BY4457 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 hos3ΔΔANΔ | This study |
| BY4458 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 rpd3ΔΔANΔ | This study |
| BY4459 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 hda1ΔHIS5 | This study |
| BY4461 | BY263 MATa cin3ΔURA3 pho85ΔLEU2 hos3ΔΔANΔ rpd3ΔΔANΔ | This study |
| BY4462 | BY2948 whi5ΔΔANΔ | This study |
| BY4463 | BY2948 hos1ΔΔANΔ | This study |
| BY4464 | BY2948 hos2ΔΔANΔ | This study |
| BY4465 | BY2948 hos3ΔΔANΔ | This study |
| BY4466 | BY2948 rpd3ΔΔANΔ | This study |
| BY4467 | BY2948 hda1ΔHIS5 | This study |
| BY4468 | BY2948 hos3ΔΔANΔ rpd3ΔΔHIS5 | This study |
| BY4541 | BY263 pho85ΔLEU2 whi5ΔΔANΔ | This study |
| BY4542 | BY263 cin3ΔTRP1 whi5ΔΔANΔ | This study |
| BY4741 | MATa leu2ΔΔ his3ΔΔ ura3ΔΔ met15ΔΔ | — |
| Y2454 | MATa mfa1Δ MFAPr-HIS3 can1Δ his3ΔΔ leu2ΔΔ lys2ΔΔ | [83] |

Of the wt strains used in this study, both BY263 and BY4741 are derived from S288C background. All the other strains are derived from these two strains. BY263 is an ssd1-d strain; BY4741 is an SSD1-V strain and is the parent strain for the yeast deletion consortium. Y2454 is congenic to BY4741 and is the parent for query strains used in synthetic genetic array (SGA) experiments.

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Kinase Assays

The in vitro protein kinase assays monitored the incorporation of [32P] transferred from γ-32P-ATP to purified recombinant GST-Whi5. The reaction mixture for assays shown in Figure 2A contained 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 10 mM MgCl2, and 1 μM ATP (including 20 μCi γ-32P-ATP) and 0.2 μg GST-Whi5 in 20 μl of total volume. 2 μl of a purified recombinant kinase (0.4 μg - 0.8 μg) was added to the mixture and incubated at 30°C for 30 min. Purification of Cln and Pcl CDKs from insect cell expression systems have been previously described [13,46]. Whi5 was then analyzed by SDS-PAGE and autoradiography. Kinase assays on immunoprecipitated proteins from yeast cell extracts were performed as described [13]. Kinase assays preceding the Whi5-SBF dissociation assay (Figure 7) were performed as described above except that 200 μM γ-32P-ATP was used instead of 1 μM. The final concentration of Cln3 and Pcl9 was 3 μM, and the final concentration of Cln2 was 60 nM (50-fold less).

Quantitative β-Galactosidase Assays

Liquid β-galactosidase assays were performed as described [29]. Strains carrying appropriate plasmids were grown in synthetic minimal medium to mid-log phase, transferred to synthetic galactose medium, and incubated for 4 h. Cells were harvested and broken in lysis buffer [100 mM Tris-HCl (pH 8.0), 1 mM DTT, and 20% glycerol with protease inhibitors] with glass beads. The β-galactosidase activity was determined by adding 100 μl of total cell extract to 0.9 ml of Z buffer (100 mM Na2HPO4, 40 mM NaHPO4, 10 mM KCl, 1 mM MgSO4, and 0.027% β-mercaptoethanol) and 200 μl ONPG (4 mg/ml) (Sigma). Units of β-galactosidase activity were determined as described [29].

Whi5 Dissociation with SBF Complex In Vitro

The protein binding assay essentially followed the procedures described previously [13]. Briefly, 1 μl of insect cell lysate expressing SBF (Swi6-Swi4FLAG) was mixed with 1 μl of purified GST-Whi5 (~0.1 μg) and 7 μl of M2 anti-FLAG resin (Sigma) in 8 μl of kinase buffer (50 mM Tris-HCl [pH 7.5], 1 mM DTT, and 10 mM MgCl2). The mixture was incubated at 4°C for 1 h with mixing. The beads bound to the SBF-Whi5 complex were then washed three times with kinase buffer, and mixed with various cyclin dependent kinases in kinase buffer with 0.2 mM ATP in a 20 μl volume. The kinase reaction was incubated at 30°C for 1 h. The soluble portion was taken out and mixed with 20 μl of 2×SDS-PAGE loading buffer. The beads in the tube were washed three times with kinase buffer before mixing with 15 μl of 2×SDS-PAGE loading buffer.

Liquid Growth Assays

Strains containing galactose-inducible plasmids were grown to saturation in 2% raffinose media for 48 h. Expression of plasmids were induced by transferring into 2% raffinose 2% galactose media and liquid growth assays were performed as previously described over 36 h using a Tecan GENios microplate reader (Tecan) [79]. Average doubling (AveG) for each culture was determined over 36 h using a Tecan GENios microplate reader [79]. Average doubling (AveG) for each culture was calculated as previously described [79]. Growth rate for each mutant was calculated relative to the AvgG of the wt strain.
Whi5-GFP Localization

The localization of Whi5-GFP was monitored in wt, cdc28Δ-4, and pho85Δ strains. Cells expressing pMET-GFP-WHI5 were grown to log phase in synthetic glucose medium without methionine. Cells were observed at a magnification of 1,000× using Nomarski optics and fluorescence microscopy and photographed by a Cascade 512B high-speed digital camera (Roemer Scientific) mounted on a Leica DM-ILB microscope. Images were captured and analyzed by MetaMorph software (Universal Imaging Media).

Table 2. Plasmids used in this study.

| Name    | Relevant Genotype | Source        |
|---------|-------------------|---------------|
| pEG-H   | pGAL1-GST URA3 2 μm | M. Snyder    |
| pMT3164 | pGAL-c-FLAG LEU2 CEN | Y. Ho        |
| pMT3446 | GST-WHS in pGEX4T1 (Escherichia coli expression vector) | M. Tyers |
| pMT3586 | pGAL-WHS-FLAG LEU2 CEN | Y. Ho        |
| pBA230v | pGPD TRP1 2 μm      | M. Funk      |
| pBA1820 | pGPD-HA-PCL1 LEU2 2 μm | This study |
| pBA1821 | pGPD-HA-PCL2 LEU2 2 μm | This study |
| pBA1822 | pGPD-HA-PCL9 LEU2 2 μm | This study |
| pBA1823 | pGPD-HA-PHO80 LEU2 2 μm | This study |
| pBA1973 | GST-WHS in pEG-H | M. Snyder |
| pBA1974 | pGAL-PCL9-FLAG LEU2 CEN | Y. Ho        |
| pBA1975 | pMET-GST-WHS HIS3 CEN | This study |
| pBA1976 | pGAL-BLXLeasp LacZ URA3 2 μm | This study |
| pBA1977 | pGPD-LexA TRP1 2 μm | This study |
| pBA1978 | pGPD-LexA-WHS TRP1 2 μm | This study |
| pBA1979 | pGPD-LexA-WHS(MBT) TRP1 2 μm | This study |
| pBA1980 | pGPD-WHS TRP1 2 μm | This study |
| pBA1981 | pMET-WHS-GFP HIS3 CEN | This study |
| pBA2112 | pGAL-HA-PCL9 URA3 2 μm | J. Moffat |
| pBA2229 | GST-PCL1 in pGEX4T1 (E.coli expression vector) | This study |
| pBA2240 | GST-PCL2 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2241 | GST-PCL3 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2242 | GST-PHO80 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2243 | GST-PHO85 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2244 | GST-CLN2 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2245 | GST-CLN3 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2246 | GST-CD28 in pAcGHIL (baculovirus transfer vector) | This study |
| pBA2247 | pGAL-CLN2-FLAG LEU2 CEN | Y. Ho        |
| pBA2248 | pGAL-CLN3-FLAG LEU2 CEN | Y. Ho        |
| pBA2249 | pMET-GST-WHS2ΔA HIS3 CEN | This study |

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