

The E2F functional analogue SBF recruits the Rpd3(L) HDAC, via Whi5 and Stb1, and the FACT chromatin reorganizer, to yeast G1 cyclin promoters

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Regulation of the *CLN1* and *CLN2* G1 cyclin genes controls cell cycle progression. The SBF activator binds to these promoters but is kept inactive by the Whi5 and Stb1 inhibitors. The Cdc28 cyclin-dependent kinase phosphorylates Whi5, ending the inhibition. Our chromatin immunoprecipitation (ChIP) experiments show that SBF, Whi5 and Stb1 recruit both Cdc28 and the Rpd3(L) histone deacetylase to *CLN* promoters, extending the analogy with mammalian G1 cyclin promoters in which Rb recruits histone deacetylases. Finally, we show that the SBF subunit Swi6 recruits the FACT chromatin reorganizer to SBF- and MBF-regulated genes. Mutations affecting FACT reduce the transient nucleosome eviction seen at these promoters during a normal cell cycle and also reduce expression. Temperature-sensitive mutations affecting FACT and Cdc28 can be suppressed by disruption of *STB1* and *WHI5*, suggesting that one critical function of FACT and Cdc28 is overcoming chromatin repression at G1 cyclin promoters. Thus, SBF recruits complexes to promoters that either enhance (FACT) or repress (Rpd3L) accessibility to chromatin, and also recruits the kinase that activates START.

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Introduction

Cell cycle progression is tightly regulated, particularly at critical points such as the transition from G1 to S phase. In metazoans, the E2F family of heterodimeric transcription factors bind to the promoters of genes that drive progression of the cell cycle (Iaquinta and Lees, 2007; van den Heuvel and Dyson, 2008). E2F binds to promoters throughout the cell cycle, but during most of the cell cycle it is kept in an inactive form due to the binding of the Rb repressor protein. Rb represses transcription in part by masking activity of

the E2F activation domain, and in part by recruiting histone deacetylases (HDAC) that modify histones at the promoters. The rise in cyclin-dependent kinase (CDK) activity during late G1 results in phosphorylated Rb, which no longer binds to E2F. This ends the recruitment of the inhibitory HDAC and exposes the E2F activation domain, allowing expression of genes such as cyclins that drive cell cycle progression.

In *Saccharomyces cerevisiae*, expression of the *CLN1* and *CLN2* G1 cyclin genes drives the transition from G1 to S known as START (Wittenberg and Reed, 2005; Bloom and Cross, 2007). *CLN1* and *CLN2* expression is activated by the heterodimeric SBF DNA-binding factor composed of Swi4 and Swi6, and SBF participates in a positive feedback loop that is important for producing the burst of cyclins that accompanies START (Skotheim *et al.*, 2008). Swi6 is also present, with Mbp1, in the heterodimeric MBF factor that promotes expression of other G1/S genes. Swi6 is present in the nucleus only during the G1 phase of the cell cycle (Sidorova *et al.*, 1995; Harrington and Andrews, 1996; Koch *et al.*, 1996).

There are strong analogies between metazoans and yeast in terms of regulation of the G1 cyclin genes. In both cases, there are heterodimeric DNA-binding activators, E2F or SBF, which are kept inactive by an inhibitor, either Rb or Whi5 in yeast (Schaefer and Breeden, 2004). Interestingly, E2F and SBF recognize very similar sequences, GCGCGAAA (Thalmeier *et al.*, 1989) and CNCGAAA (Badis *et al.*, 2008), even though they are unrelated by sequence or structural motifs. In both cases, gene activation requires a CDK (Cdc28 in yeast) that phosphorylates and inactivates the inhibitor (Costanzo *et al.*, 2004; de Bruin *et al.*, 2004). Phosphorylation of the Whi5 inhibitor in yeast results in export from the nucleus, and this is an important part of the positive feedback loop that drives the cell cycle (Skotheim *et al.*, 2008).

The Stb1 protein also contributes to the regulation of SBF and MBF target genes. Stb1 interacts with SBF and MBF, and Stb1 binds to SBF- and MBF-specific promoters during G1 phase (Ho *et al.*, 1999; Costanzo *et al.*, 2003; de Bruin *et al.*, 2008). Stb1 has a function in both transcriptional repression and activation, as *stb1* mutants reduce both the repression in early G1 and also the induced RNA level of late G1 (de Bruin *et al.*, 2008). Stb1 has been shown to interact with Sin3, a component of Rpd3(L) (Kasten and Stillman, 1997), and also with Swi6, present in both SBF and MBF (Ho *et al.*, 1999).

Activation of the yeast *HO* gene requires a number of activators, including SBF (Breeden and Nasmyth, 1987; Nasmyth, 1993). We have recently described alterations in chromatin structure that occur at the *HO* promoter during the cell cycle, and showed that changes at the URS2 region of the promoter require SBF (Takahata *et al.*, 2009). SBF is required both for eviction of nucleosomes and for the recruitment of three coactivator complexes, Swi/Snf, SAGA and Mediator, to *HO* URS2. Additionally, SBF recruits the FACT chromatin

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reorganizing complex to the promoter, and FACT and Swi6 co-immunoprecipitate (Takahata *et al*, 2009). The FACT chromatin reorganizing complex changes the accessibility of DNA within a nucleosome, but unlike Swi/Snf remodelers, FACT activity is ATP independent (Formosa, 2008). Genetic and biochemical studies suggest FACT functions in DNA replication, transcription initiation, and transcription elongation. We recently showed that FACT mutations lower *HO* expression by reducing nucleosome eviction from the region of the *HO* promoter bound by SBF (Takahata *et al*, 2009). FACT mutants are defective in expressing G1 cyclin genes and show impaired G1/S transition (Rowley *et al*, 1991; Lycan *et al*, 1994; Schlesinger and Formosa, 2000; Biswas *et al*, 2008b).

Here, we investigate events at the promoters of the *CLN1* and *CLN2* genes. We show that SBF binds transiently during the cell cycle to these promoters, and that SBF recruits Cdc28, Rpd3(L) and FACT to these promoters. FACT binds transiently to the promoters of the G1/S regulon genes, and FACT mutations reduce both nucleosome eviction and gene transcription. We extend the analogy between yeast and metazoan cyclin genes by showing that the Rpd3(L) HDAC is recruited to the promoters of the yeast G1 cyclin genes by SBF. Rpd3(L) recruitment requires the Whi5 inhibitor and also the Stb1 protein. Stb1 has been shown to interact with Sin3, a component of Rpd3(L) (Kasten and Stillman, 1997), and also with Swi6 (Ho *et al*, 1999). Finally, mutations in *STB1* and *WHI5* permit growth at normally nonpermissive temperatures of strains with conditional mutations affecting FACT and Cdc28, suggesting that transcriptional activation of G1/S target genes by overcoming Stb1/Whi5 inhibition is a critical function of FACT and Cdc28.

Results

SBF binds to *CLN2* transiently during the cell cycle

We used ChIP assays to measure Swi4-Myc binding to the *CLN2* promoter during the cell cycle (Figure 1A). Cells were synchronized by *GALp::CDC20* arrest and release, and samples were taken at various times to determine SBF binding. SBF binding begins at ~20 min after release and peaks at 50 min. Late G1 in these synchronized cells is about 30 min, when *CLN2* mRNA levels start to rise; *CLN2* RNA levels peak at about 50 min. Using synchronized cells, we see SBF binding to the *CLN1* and *CLN2* promoters during G1, but binding is not seen in G2-arrested cells (Figure 1B). SBF does not bind to *CDC21*, an MBF-activated gene. SBF association with the cyclin promoters is therefore transient and correlates with transcription of the genes.

Rpd3(L) is recruited to SBF- and MBF-dependent promoters by Swi6

SBF is functionally analogous to mammalian E2F, and E2F recruits HDACs to promoters (Iaquinta and Lees, 2007; van den Heuvel and Dyson, 2008). On the basis of this analogy, we asked whether the Rpd3(L) HDAC was recruited to the *CLN2* promoter. We performed ChIP assays with synchronized cells to detect Sds3-Myc at promoters. Sds3 is a subunit of Rpd3(L), but absent from the Rpd3(S) complex (Carozza *et al*, 2005). Rpd3(L) and Rpd3(S) appear to have different functions, with Rpd3(L) localized primarily to promoter regions and Rpd3(S) at transcribed regions (Joshi and Struhl, 2005). Rpd3(L) binds to *CLN2* with kinetics very

similar to that observed for SBF (Figure 1A). However, Rpd3(L) binding decays more rapidly than does SBF. The similar kinetics of binding for SBF and Rpd3(L) suggest that SBF recruits Rpd3(L). To test this idea we examined Rpd3(L) binding to promoters in *swi6* mutants (Figure 1C). Binding of the Sds3-Myc subunit of Rpd3(L) to *CLN1* and *CLN2* is lost in the *swi6* mutant. Rpd3(L) also binds to the promoter of the MBF-dependent *CDC21* gene, although later in the cell cycle. Swi6 is a subunit of both SBF and MBF, and Rpd3(L) binding to *CDC21* is also lost in the *swi6* mutant. Rpd3(L) also binds to the *CTS1* promoter, recruited by the Fkh1 and Fkh2 factors (Voth *et al*, 2007). No defect in Rpd3(L) binding to *CTS1* is seen in the *swi6* mutant, and thus the *swi6* mutation does not simply affect the integrity of the HDAC complex.

Whi5 and Stb1 are required for Rpd3(L) binding to SBF-dependent genes

Stb1 and Whi5 interact with SBF and are described as inhibitors of SBF (Costanzo *et al*, 2004; de Bruin *et al*, 2004, 2008). The analogy between these inhibitors and mammalian Rb protein suggested that they could be the intermediaries that allow SBF to recruit the Rpd3(L) HDAC. We examined Rpd3(L) binding to the *CLN1* and *CLN2* promoters in strains with either a *stb1* or a *whi5* mutation (Supplementary Figure S1). A *stb1* mutation modestly reduces Rpd3(L) binding to *CLN1* or *CLN2*, and a *whi5* mutation has little effect on Rpd3(L) binding. We next examined Rpd3(L) binding to SBF-dependent promoters in a strain lacking both Stb1 and Whi5 (Figure 2A). Rpd3(L) binding to the SBF-dependent *CLN1* and *CLN2* promoters is essentially eliminated in the *stb1 whi5* mutant. Importantly, Rpd3(L) binding to *CTS1* is not affected in the *stb1 whi5* double mutant (Figure 2C). Additionally, SBF binding to *CLN1* and *CLN2* is not affected by either a *stb1* or a *whi5* mutation (Supplementary Figure S2). We conclude that Whi5 and Stb1 both link Rpd3(L) to SBF.

Whi5 has been described as a negative regulator of SBF-dependent genes (Costanzo *et al*, 2004; de Bruin *et al*, 2004), consistent with its ability to recruit Rpd3(L). This predicts that a *whi5* mutation should result in increased expression of target genes. However, RNA measurements show nearly normal expression of *CLN1* and *CLN2* in *whi5* mutants (Figure 2D). Stb1 has been described as promoting transcriptional activation (de Bruin *et al*, 2008), and consistent with this, we find decreased expression of SBF-dependent genes in a *stb1* mutant (Figure 2D). Interestingly, the *stb1* defect in transcriptional activation is suppressed by a *whi5* mutation. As a control, expression of the *CYC1* gene is unaffected by *whi5* or *stb1* mutations (Supplementary Figure S3). These results support the idea that Stb1 is an activator, whereas Whi5 is a transcriptional inhibitor, but additional mechanisms appear to limit the level of *CLN1* and *CLN2* transcription to near the WT level in cells lacking only Whi5.

We expected that *CLN1* and *CLN2* RNA levels would be significantly higher in the *stb1 whi5* double mutant, as Rpd3(L) binding to these promoters is lost in this mutant. However, in the asynchronous *stb1 whi5* cells *CLN2* RNA was at wild-type levels and *CLN1* RNA was only slightly increased (Figure 2D). We therefore synchronized wild-type and *stb1 whi5* cells and measured *CLN1* and *CLN2* RNA during the cell cycle (Figure 2E). There is no significant effect on the kinetics or RNA accumulation, and only a modest increase in the levels of *CLN1* and *CLN2* RNA.

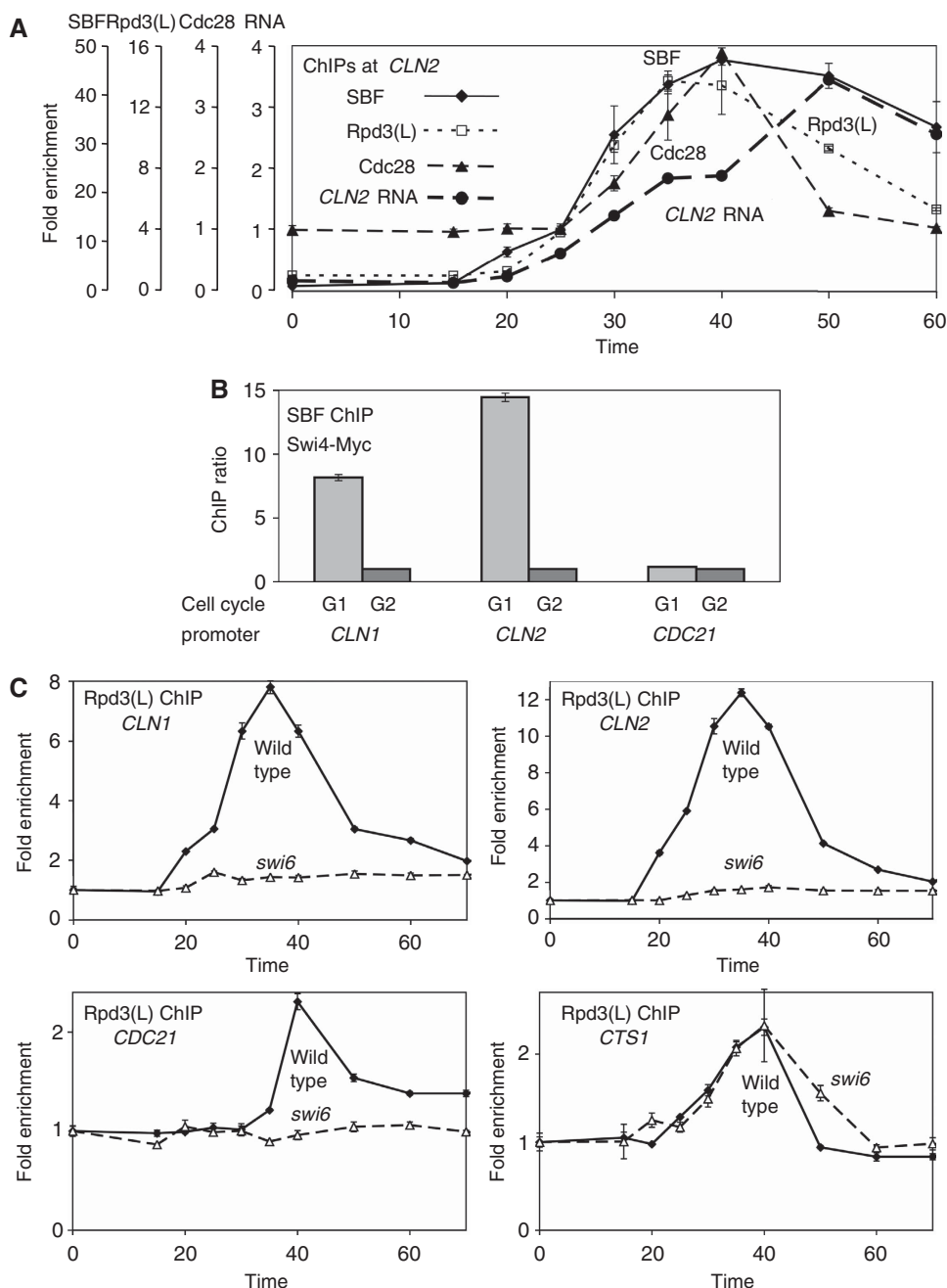


Figure 1 SBF recruits Rpd3(L) to promoters. (A) DY12794 (*GALp::CDC20 SWI4-Myc*), DY12247 (*GALp::CDC20 SDS3-Myc*) and DY6669-Cdc28-HA (*GALp::CDC20 YCp::KanMX::CDC28-HA*) cells were synchronized in mitosis by removing galactose, followed by release by addition of galactose (0 min). The DY6669-Cdc28-HA cells were grown in medium containing G418. The *CDC20* arrest is at the G2/M transition, and *CLN2* expression begins at 30 min after release corresponds to late G1 phase. Binding of the Swi4-Myc subunit of SBF and the Sds3-Myc subunit of Rpd3(L) were measured ChIP using samples taken at various times after the release. *CLN2* RNA measurements were from synchronized DY12752 (*GALp::CDC20 GCN5-Myc*). (B) Swi4-Myc binding at *CLN1* (SBF regulated), *CLN2* (SBF regulated) and *CDC21* (MBF regulated) was assessed in DY12794 cells (*GALp::CDC20 SWI4-Myc*) either in G1 (35 min after *CDC20* arrest and release) or arrested in G2 (0 min). (C) Strains DY12247 (*GALp::CDC20 SDS3-Myc*) and DY12828 (*GALp::CDC20 SDS3-Myc swi6*) were synchronized and ChIP experiments performed to measure Rpd3(L) binding at *CLN1*, *CLN2*, *CDC21* and *CTS1*. A full-colour version of this figure is available at *The EMBO Journal* Online.

Only *Stb1* is required for Rpd3(L) binding to MBF-dependent genes

Rpd3(L) binds to the promoters of MBF-dependent genes, and this binding requires the Swi6 protein common to both SBF and MBF (Figure 1C). Similarly, Rpd3(L) binding to *CDC6* and *CDC21*, which are MBF dependent, is lost in the *stb1 whi5* double mutant (Figure 2B), like the SBF-dependent *CLN1* and

CLN2 promoters. Rpd3(L) binding to MBF target genes is not affected by a *whi5* mutation (Supplementary Figure S1C), similar to SBF genes (Supplementary Figure S1A). However, a major difference in factor dependence was seen for Rpd3(L) binding to MBF-dependent and SBF-dependent promoters. A *stb1* mutation essentially eliminates Rpd3(L) binding to *CDC6* and *CDC21* (Supplementary Figure S1D), whereas the

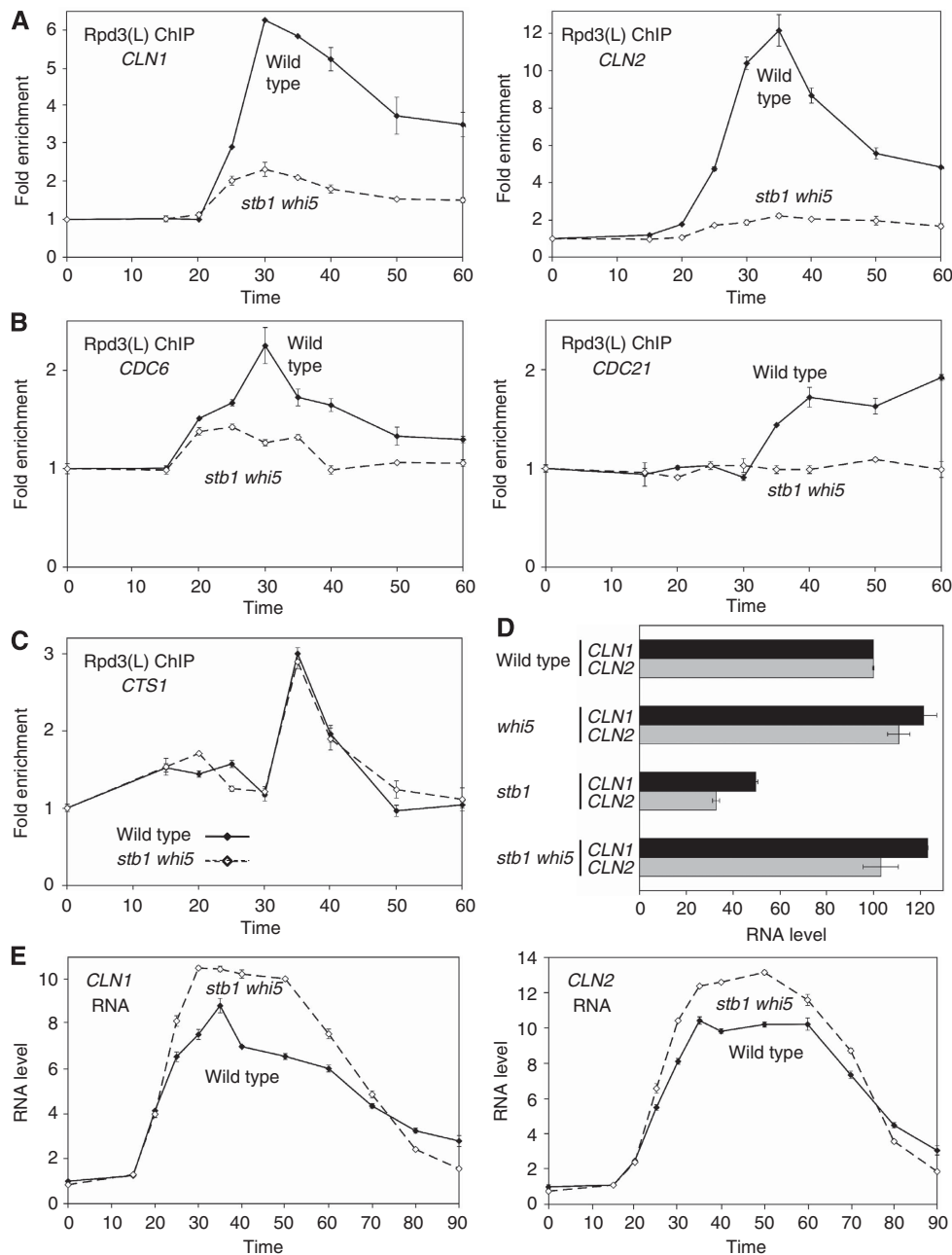


Figure 2 Rpd3(L) binding to SBF and MBF promoters requires Stb1 and Whi5. (A–C) Strains DY12248 (*GALp::CDC20 SDS3-Myc*) and DY13502 (*GALp::CDC20 SDS3-Myc stb1 whi5*) were synchronized and ChIP experiments performed to measure Rpd3(L) binding at (A) SBF-dependent *CLN1* and *CLN2*, (B) MBF-dependent *CDC6* and *CDC21* and (C) SBF- and MBF-independent *CTS1*. (D) *CLN1* and *CLN2* expression is reduced in *stb1* mutants but not in *stb1 whi5* double mutants. RNA was isolated from log phase wild-type (DY150), *whi5* (DY10474), *stb1* (DY13454) and *stb1 whi5* (DY13494), strains and mRNA levels measured by RT-qPCR. (E) *CLN1* and *CLN2* expression is modestly increased in synchronized *stb1 whi5* double mutants. Strains DY6669 (*GALp::CDC20*) and DY13496 (*GALp::CDC20 stb1 whi5*) were synchronized and mRNA levels measured by RT-qPCR.

stb1 mutation results in only a modest reduction in Rpd3(L) binding to the SBF-dependent *CLN1* and *CLN2* promoters (Supplementary Figure S1B). Expression of MBF-dependent genes is reduced in a *stb1* mutant (Supplementary Figure S3), as was seen for SBF-dependent genes (Figure 2D). However, although a *whi5* mutation strongly suppresses the *stb1* defect in the activation of MBF-dependent genes, there is less suppression at MBF-dependent genes by *whi5*.

Although Stb1 interacts with the Swi6 subunit present in both SBF and MBF (de Bruin *et al*, 2008), there is discrepancy

in the literature as to whether Whi5 interacts with both SBF and MBF or with SBF alone (Costanzo *et al*, 2004; de Bruin *et al*, 2004). To address this question in our strain background we performed co-immunoprecipitation experiments with tagged alleles. The experiment performed with extracts prepared from a Whi5-Flag and Swi4-Myc strain show that Whi5 interacts with the SBF-specific Swi4 protein, irrespective of which antibody is used for the immunoprecipitation (Supplementary Figure S4A). In contrast, we saw no interaction between Whi5-Flag and the Mbp1-Myc subunit of

MBF (Supplementary Figure S4B). We conclude that Whi5 does not interact with MBF, and this is consistent with our result that only Stb1 is required for recruiting Rpd3(L) to MBF-dependent genes.

***Cdc28* is recruited to SBF- and MBF-dependent promoters**

The *Cdc28* CDK has long been implicated in transcriptional activation by SBF and MBF (Wittenberg and Reed, 2005). *Cdc28* has been shown to be directly recruited to the *CDC20* promoter when it is expressed in late G2 (Morris *et al*, 2003), so we performed ChIP experiments and found that *Cdc28* is also recruited directly to both SBF- and MBF-dependent promoters (Supplementary Figure S5). Disruption of either *SWI6* or *WHI5* eliminates *Cdc28* binding, whereas a *stb1* mutation only modestly reduces *Cdc28* recruitment. Whi5 does not bind to MBF (Supplementary Figure S4; de Bruin *et al*, 2004), and thus it is surprising that *Cdc28* binding is reduced in the *whi5* mutant. A *whi5* mutation affects cell cycle progression (Jorgensen *et al*, 2002), and thus it could indirectly affect *Cdc28* recruitment in these asynchronous cells. *CDC20* expression is independent of SBF or MBF (Simon *et al*, 2001), and *Cdc28* recruitment to *CDC20* is not affected by *swi6*, *whi5* or *stb1* mutations. We also examined *Cdc28* binding to *CLN2* in synchronized cells (Figure 1A). The kinetics of *Cdc28* binding is similar to that seen for SBF, although *Cdc28* binding decreases more rapidly after peak binding. This rapid decline in *Cdc28* binding is consistent with this kinase phosphorylating Whi5 resulting in nuclear export of Whi5 (Costanzo *et al*, 2004; de Bruin *et al*, 2004), and the loss of Whi5 would end *Cdc28* binding. We conclude that *Cdc28* recruitment to SBF- and MBF-dependent promoters requires Swi6 and Whi5.

FACT and Swi6 are required for nucleosome eviction at *CLN2*

Chromatin disassembly at promoters often accompanies gene activation (Workman, 2006; Williams and Tyler, 2007). We determined earlier that nucleosome eviction at the SBF-dependent *HO* promoter requires both FACT and Asf1 histone chaperones (Takahata *et al*, 2009). FACT co-immunoprecipitates with Swi6 (Takahata *et al*, 2009), and we wanted to determine whether this interaction was direct. We expressed a GST-Swi6 fusion protein in *Escherichia coli*, and loaded the purified GST-Swi6 protein onto glutathione-agarose beads. Purified FACT was loaded onto the beads, washed and eluted. The Spt16 and Pob3 subunits of FACT bind to GST-Swi6, but not to the GST-only or the GST-Swi5 controls (Figure 3). We conclude that FACT and Swi6 interact directly.

We next examined nucleosome eviction from cyclin gene promoters during their activation, and any function for FACT and Asf1 in this process. We used synchronized cells for a ChIP experiment to measure histone H3 occupancy at *CLN2* during the cell cycle in wild-type cells and in cells with mutations in *ASF1*, *SWI6* and *POB3* (Figure 4A). The Spt16 and Pob3 subunits of FACT are encoded by essential genes (Formosa, 2008), and in these experiments we used a *pob3(L78R)* mutation that strongly decreases the level of Pob3 protein even at the permissive temperature (VanDemark *et al*, 2008). In wild-type cells, nucleosomes are transiently evicted from the *CLN2* promoter at 30 min after release from *GALp::CDC20* arrest. Nucleosome eviction

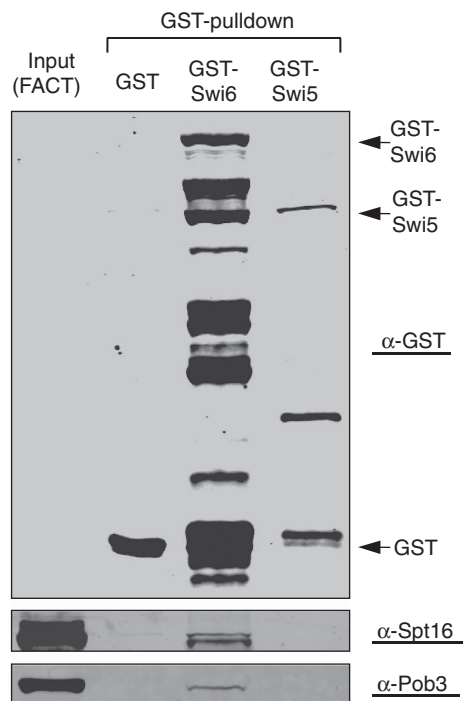


Figure 3 FACT interacts directly with Swi6. Glutathione-agarose beads were loaded with either GST only, GST-Swi6 or GST-Swi5 and purified FACT was applied to the beads, washed and eluted. The eluted material is analysed on western blots with antibodies to GST, Spt16 and Pob3. There are significant degradation products, particularly for GST-Swi6. Lane 1 contains the input extract (corresponding to 50% of the amount of material applied to the beads).

at *CLN2* is sharply reduced in *pob3* or *swi6* mutants, but the *asf1* mutation has no effect. Thus, *CLN2* differs from *PHO5* and *HO* in which Asf1 is required for chromatin disassembly (Adkins *et al*, 2004; Takahata *et al*, 2009). *PIR1* is a cell cycle-regulated gene expressed in M/early G1, and nucleosomes are evicted from the *PIR1* promoter transiently during the cell cycle (Figure 4B). The *pob3* and *swi6* mutations have no effect on nucleosome loss at *PIR1*. Our data suggest that chromatin disassembly occurs at many cell cycle genes coincident with transcriptional activation, but the mechanism of eviction varies widely. Importantly, FACT and Swi6 are required for the changes in chromatin structure at the *CLN2* promoter.

Nucleosome eviction at *CLN2* is reduced, or possibly delayed, in strains with mutations affecting FACT or SBF. On the basis of this result, we examined *CLN2* mRNA levels during the cell cycle in synchronized wild-type, *pob3* or *swi6* strains. There is a marked delay in the appearance of *CLN2* mRNA in the *pob3* and *swi6* mutants (Supplementary Figure S6A), but transcription eventually occurs. Earlier work has suggested a function for Rme1 in activating *CLN2* (Toone *et al*, 1995). Overexpression of Rme1 increases *CLN2* expression and also permits growth of normally inviable cells lacking both SBF subunits. The Rme1 DNA-binding protein binds to the *CLN2* promoter *in vitro* and activates through promoter sequences distinct from the SBF-binding sites. To address the function of *RME1* in *CLN2* activation, we constructed *rme1* mutant strains and also made double mutants with *swi6* and *pob3*. RNA measurements with

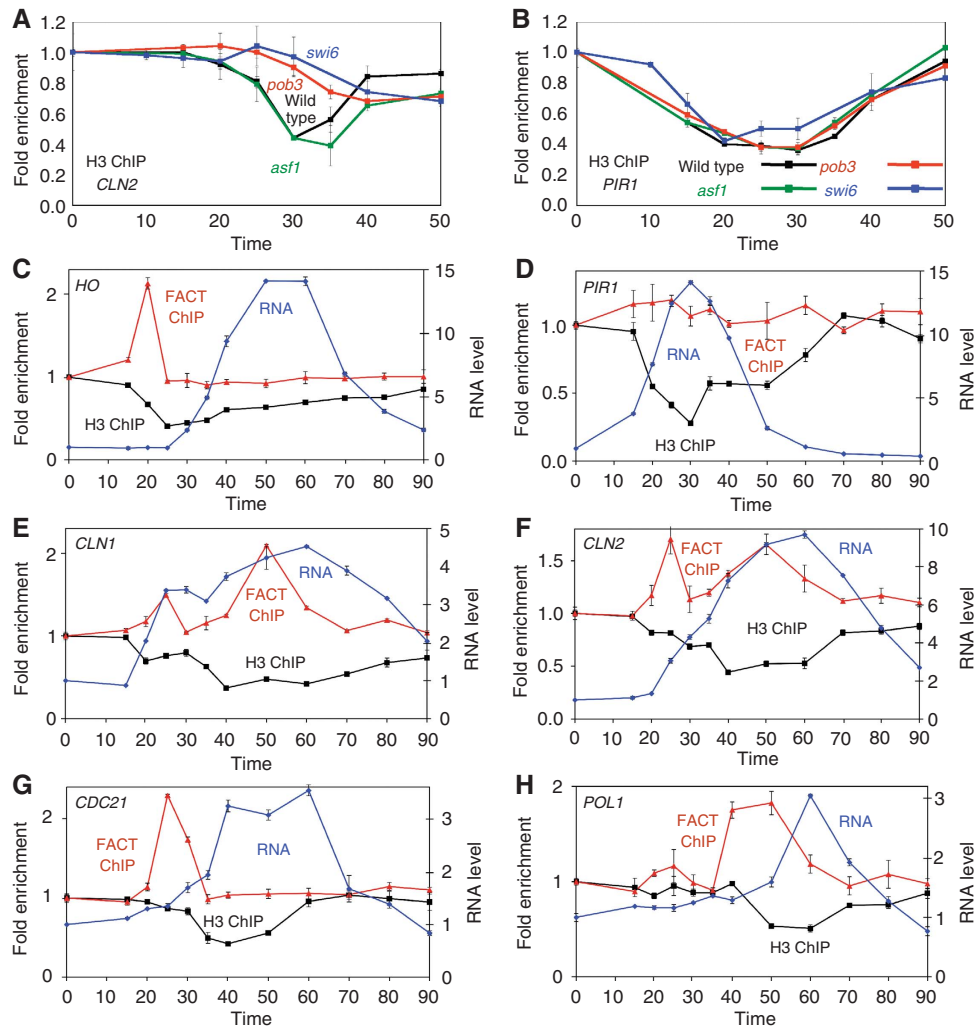


Figure 4 FACT binding at SBF and MBF promoters precedes nucleosome eviction and gene expression. (A) Histone H3 ChIP was performed to measure nucleosome occupancy at the *CLN2* promoter using synchronized strains DY6669 (*GALp::CDC20*), DY12914 (*GALp::CDC20 asf1*), DY11246 (*GALp::CDC20 pob3*) and DY13373 (*GALp::CDC20 swi6*). (B) Histone H3 ChIP measuring nucleosome occupancy at the *PIR1* promoter, as in panel A. (C–H) Strain DY6669 (*GALp::CDC20*) was synchronized and samples taken for FACT (Spt16) ChIP, histone ChIP and mRNA measurements for (C) *HO* URS2, (D) *PIR1*, (E) *CLN1*, (F) *CLN2*, (G) *CDC21* and (H) *POL1*. Supplementary Figure S8 shows the same experiment conducted with a *swi6* mutant.

asynchronous cells show reduced *CLN2* expression in *rme1* mutants, and strong additive defects in *swi6 rme1* and *pob3 rme1* double-mutant strains (Supplementary Figure S7). Although the *swi6* and *pob3* mutations reduce *HO* expression, little additive effect was seen in the *swi6 rme1* and *pob3 rme1* double-mutant strains. Finally, *PIR1* expression was unaffected by *rme1*, *swi6* or *pob3* mutations. These results are consistent with the idea that Rme1 promotes the residual *CLN2* activation in strains with SBF or FACT mutations.

In contrast to the situation at *CLN2* in which there is residual expression in the *swi6* and *pob3* mutants (Supplementary Figure S6A), expression of the SBF- and FACT-dependent *HO* gene is largely eliminated in both *pob3* and *swi6* cells (Supplementary Figure S6B). *HO* gene transcription requires recruitment of coactivator complexes to the URS2 region of the promoter, and mutations affecting FACT or SBF eliminate this coactivator recruitment (Takahata *et al*, 2009). We therefore examined binding of the Gcn5 subunit of the SAGA coactivator to *CLN2* using ChIP assays to see whether this mechanism is also used at this promoter

(Supplementary Figure S6C). In wild-type cells Gcn5 binds transiently to *CLN2* during the cell cycle, at about the same time as SBF that binds. The *pob3* and *swi6* mutations do not eliminate Gcn5 binding to *CLN2*, but rather cause a delay similar to the delay in mRNA levels. We conclude that FACT and Swi6 are required to express the *CLN2* promoter at the appropriate time because they allow eviction of nucleosomes and coactivator recruitment to occur rapidly. This is unlike the situation at the *HO* promoter in which the gene is completely transcriptionally inactive in FACT or SBF mutants. In contrast, nucleosome eviction, coactivator recruitment and gene expression eventually occur at *CLN2* in these mutants, and thus FACT and Swi6 are not absolutely required here.

Swi6 is required for FACT binding and nucleosome eviction at SBF- and MBF-regulated genes

On the basis of the delay in *CLN2* activation in *swi6* mutants during the cell cycle, we decided to repeat the histone H3 ChIP experiment but with a longer time course, so that we could discriminate between events that are required for *CLN2*

activation and those that affect its rate. FACT binds to *HO* URS2 in a *SWI6*-dependent manner (Takahata *et al*, 2009), and so we measured FACT binding in wild-type cells (Figure 4C–H), and in *swi6* mutants (Supplementary Figure S8). Both wild-type and *swi6* cells were synchronized by *GALp::CDC20* arrest and release, and samples were taken at time points for H3 ChIP, FACT ChIP and RNA measurements; each measurement is therefore from the same culture of synchronized cells.

FACT binds to *HO* URS2 at 20 min, just before nucleosomes are evicted, with strong gene expression seen starting at 40 min (Figure 4C), consistent with our earlier experiments (Takahata *et al*, 2009). The *PIR1* gene, whose cell cycle expression is independent of Swi6, serves as a control (Figure 4D). *PIR1* expression is not affected by a FACT mutation (Takahata *et al*, 2009), and FACT does not bind to this promoter. Nucleosomes are transiently evicted during the cell cycle as the gene is expressed, and these events are not affected in a *swi6* mutant (Supplementary Figure S8). Thus, the *swi6* mutation does not disrupt events at all cell cycle-regulated genes.

Expression of the SBF-dependent genes *CLN1* and *CLN2* is reduced in FACT mutants (Rowley *et al*, 1991; Lycan *et al*, 1994; Takahata *et al*, 2009), and this is also seen in our experiments analysing RNA levels in synchronized cells (Figure 4; Supplementary Figure S8). There are two peaks of FACT binding at these promoters, one at 25 min after release and one at 50 min. (See also the independent ChIP experiments in Figure 5 showing this result is reproducible.) Nucleosome occupancy decreases first at 20 min, and then further at 40 min; return of nucleosomes to the promoters begins at 50 min. There is no FACT binding to these promoters in the *swi6* mutant (Supplementary Figure S8C and D). However, the *swi6* mutation causes only a modest change in nucleosome disassembly, despite the markedly reduced and delayed gene expression. Although no FACT binds to the promoters in the *swi6* mutant, nucleosome eviction and gene transcription still occur, albeit more slowly. The delayed and reduced residual chromatin disassembly and transcription may be induced by Rme1 (Toone *et al*, 1995). We conclude that although FACT recruitment is correlated with timely gene

induction, it is not absolutely required for nucleosome disassembly at these promoters.

We also examined promoter events at two MBF-dependent promoters, *CDC21* and *POL1*. At *CDC21* there is a sharp peak of FACT binding at 20 min, followed by nucleosome loss and gene transcription (Figure 4G). All of these events are eliminated in a *swi6* mutant (Supplementary Figure S8E). The kinetics at *CDC21* resemble those seen at *HO* (Figure 4C), except that FACT binding and chromatin disassembly occur slightly sooner at *HO*. Events at *POL1* occur later in the cell cycle (Figure 4H). Major FACT binding is seen at 40 min after release, although there may be weak transient binding at 20–25 min. Nucleosome eviction and gene transcription also occur later, after the peak in FACT binding. FACT binding, chromatin disassembly, and gene activation are all lost in a *swi6* mutant (Supplementary Figure S8F).

These studies allow us to make a number of conclusions regarding the common features of promoters that are expressed at different times during the cell cycle. FACT binds transiently during the cell cycle to promoters of SBF- and MBF-activated genes. This FACT binding is followed first by changes in chromatin structure at the promoter and subsequently by gene transcription. Swi6 is required for recruitment of FACT to these promoters and for chromatin disassembly, although there is significant nucleosome loss at *CLN1* and *CLN2* in a *swi6* mutant so FACT is not required for all nucleosome loss, and nucleosome loss is not sufficient to cause increased transcription.

Whi5 and Stb1 inhibit FACT binding to SBF-dependent promoters

SBF recruits both Stb1 and Whi5 to promoters, and SBF also recruits FACT. We wondered whether Stb1 or Whi5 were required for recruiting FACT. We measured FACT binding in synchronized wild-type cells and a *stb1 whi5* double mutant. To our surprise, in *stb1 whi5* mutants we observed markedly increased FACT binding to three SBF-dependent genes, *HO*, *CLN1* and *CLN2* (Figure 5A). This suggests that Stb1 or Whi5, or both, inhibit FACT binding to SBF-dependent promoters. In contrast, FACT binding to three MBF-dependent promoters was not affected in the *stb1 whi5* double mutants (Figure 5B).

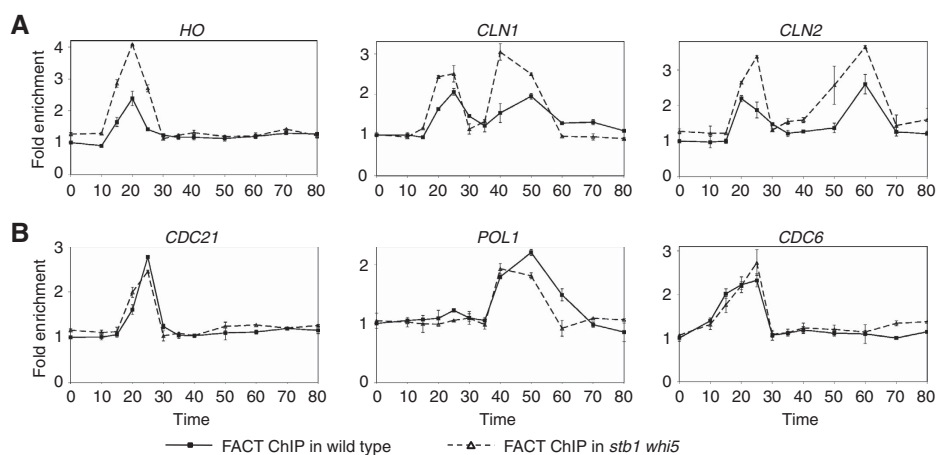


Figure 5 FACT binding to SBF-regulated genes increases in *stb1 whi5* mutant. (A) FACT binding to SBF promoters increases in a *stb1 whi5* mutant. Strains DY6669 (*GALp::CDC20*) and DY13496 (*GALp::CDC20 swi6*) were synchronized and FACT binding to SBF promoters *HO* URS2, *CLN1* and *CLN2* was measured. (B) FACT binding to MBF promoters does not change in a *stb1 whi5* mutant. The ChIP material used in panel A was analysed for FACT binding to MBF promoters *CDC21*, *POL1* and *CDC6*. A full-colour version of this figure is available at *The EMBO Journal* Online.

Whi5 binds to SBF but not to MBF (Supplementary Figure S4; de Bruin *et al*, 2004). We suggest that Whi5 antagonizes FACT binding to SBF-dependent promoters, consistent with Whi5's function as a transcriptional inhibitor.

***stb1* and *whi5* suppress FACT and *cdc28* mutations**

Consistent with an antagonistic relationship between FACT and Whi5, we identified genetic interactions between FACT, *WHI5* and *STB1*. An *spt16-11* mutation in FACT shows growth defects at higher temperatures, but this defect is suppressed by *whi5* (Figure 6A). In contrast, the *spt16-11 stb1* double mutant shows an additive defect compared with either single mutant at 35°C or on media containing hydroxyurea (HU). This defect is suppressed in the *spt16-11 stb1 whi5* triple mutant, and thus *whi5* is epistatic to *stb1*. A *whi5* mutation also suppresses both the temperature- and HU-sensitive phenotypes caused by two *pob3* alleles (Figure 6B and C); a synthetic defect is not seen in the *pob3 stb1* double mutants.

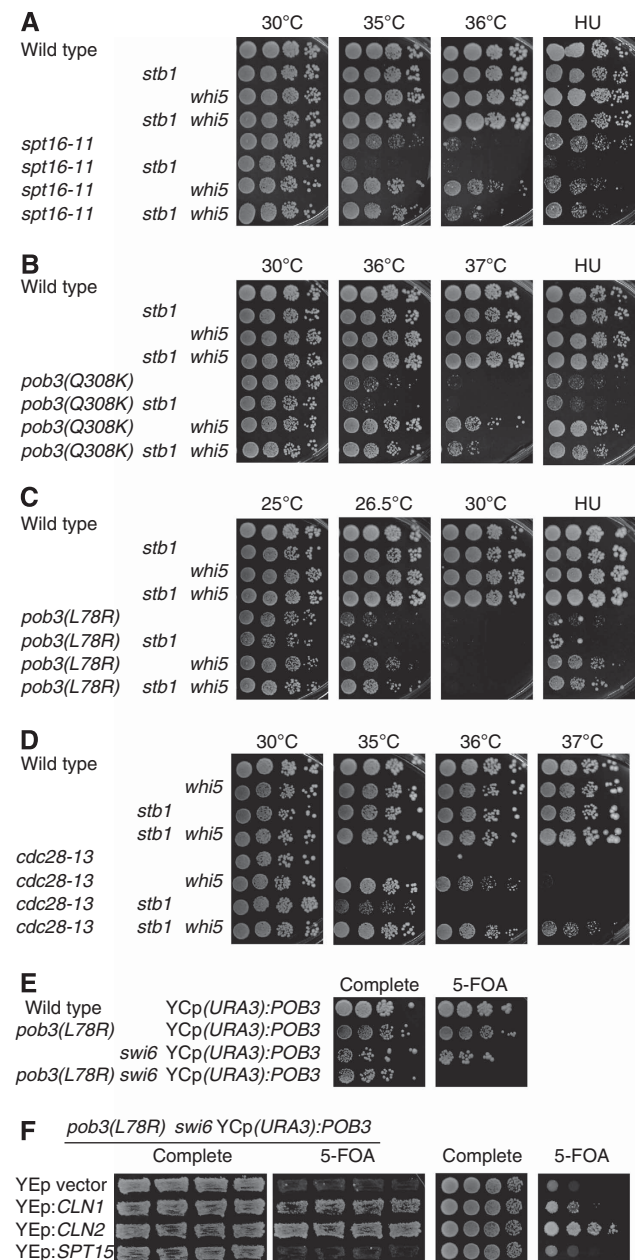
At the nonpermissive temperature, *cdc28-13* mutants arrests in G1, before START (Mendenhall *et al*, 1988). We next examined whether *stb1* or *whi5* mutations could suppress the *cdc28-13* defects. Although *cdc28-13* cells do not form colonies at temperatures of 35°C or above, growth is restored by either a *stb1* or *whi5* mutation (Figure 6D). Suppression by *whi5* is more robust, and only the *cdc28-13 whi5* strain is able to grow at 36°C, whereas *cdc28-13 stb1* cells cannot. Finally, suppression by *stb1* and *whi5* is additive, as only the *cdc28-13 stb1 whi5* triple mutant can grow at 37°C. This *stb1 whi5* double mutant has a growth defect at elevated temperatures (Supplementary Figure S9), making this suppression of

cdc28-13 all the more impressive. These genetic results confirm that Whi5 antagonizes both FACT and CDK functions, but *Stb1*'s function is more complex as it appears to promote FACT function but to antagonize CDK.

***CLN2* overexpression suppresses *pob3 swi6* synthetic lethality**

CLN2 transcription is reduced in both *pob3* and *swi6* mutants, and we wanted to see whether *CLN2* expression is further reduced in a double-mutant strain. A doubly heterozygous +/*pob3* +/*swi6* diploid strain was transformed with a YCp(*URA3*) plasmid with a wild-type *POB3* gene. After sporulation, a haploid *pob3 swi6* double-mutant strain with the YCp(*URA3*):*POB3* was isolated. We tested whether this strain could grow on medium containing 5-FOA, as only strains lacking the *URA3* plasmid can grow on these plates. The *pob3 swi6* YCp(*URA3*):*POB3* cells are unable to grow on

Figure 6 *stb1* and *whi5* suppress *cdc28* and FACT mutations. (A) Serial dilutions of strains DY150 (wild type), DY13454 (*stb1*), DY9558 (*whi5*), DY13640 (*stb1 whi5*), DY7815 (*spt16-11*), DY13714 (*spt16-11 stb1*), DY13716 (*spt16-11 whi5*) and DY13718 (*spt16-11 stb1 whi5*) were incubated on medium at 30°C for 2 days, at 35°C for 2 days, at 36°C for 3 days, or on medium containing 50 mM HU at 25°C for 5 days. (B) Serial dilutions of strains DY150 (wild type), DY13454 (*stb1*), DY9558 (*whi5*), DY13640 (*stb1 whi5*), DY12236 (*pob3(Q308K)*), DY13696 (*pob3(Q308K) stb1*), DY13698 (*pob3(Q308K) whi5*) and DY13700 (*pob3(Q308K) stb1 whi5*) were incubated on medium at 30°C for 2 days, at 36°C for 3 days, at 37°C for 3 days, or on medium containing 25 mM HU at 25°C for 3 days. (C) Serial dilutions of strains DY150 (wild type), DY13454 (*stb1*), DY9558 (*whi5*), DY13640 (*stb1 whi5*), DY7379 (*pob3(L78R)*), DY13704 (*pob3(L78R) stb1*), DY13702 (*pob3(L78R) whi5*) and DY13706 (*pob3(L78R) stb1 whi5*) were incubated on medium at 25°C for 3 days, at 26.5°C for 4 days, at 30°C for 2 days, or on medium containing 100 mM HU at 25°C for 5 days. (D) Serial dilutions of strains DY150 (wild type), DY10474 (*whi5*), DY13454 (*stb1*), DY13494 (*stb1 whi5*), DY12836 (*cdc28-13*), DY11982 (*cdc28-13 whi5*), DY13541 (*cdc28-13 stb1*) and DY13543 (*cdc28-13 stb1 whi5*) were incubated on medium at 30°C for 3 days, at 35°C for 3 days, at 36°C for 3 days or at 37°C for 5 days. (E) Serial dilutions of strains DY150 (wild type), DY7379 (*pob3(L78R)*), DY13357 (*swi6*) and DY14024 (*pob3(L78R) swi6*), each transformed with the YCp(*URA3*):*POB3* plasmid, were incubated on either complete medium or 5-FOA plates at room temperature for 4 days. (F) Strain DY14024 (*pob3(L78R) swi6*) with the YCp(*URA3*):*POB3* plasmid was transformed with one of four multicopy plasmids, the empty YEp-*LEU2* vector, YEp:*CLN1*, YEp:*CLN2*, or YEp:*SPT15*, and the transformants tested for the ability to lose the YCp(*URA3*):*POB3* plasmid and grow on 5-FOA plates. In the left two panels, four transformants were patched on medium lacking leucine and uracil, and replica plated and grown at room temperature for 4 days. In the right two panels, serial dilutions of a single transformant were incubated at room temperature for 5 days.



5-FOA (Figure 6E), showing that *pob3* and *swi6* are synthetically lethal. We next transformed these *pob3 swi6* YCp(*URA3*):*POB3* cells with multicopy YEp(*LEU2*) plasmids, and tested these transformants for the ability to grow on 5-FOA medium. As shown in Figure 6F, a multicopy plasmid with *CLN2* allows cells to lose the YCp(*URA3*):*POB3* plasmid and grow on 5-FOA; multicopy *CLN1* also suppresses the *pob3 swi6* synthetic lethality but to a lesser extent. This genetic experiment suggests that the *pob3* and *swi6* mutations are synthetically lethal because of insufficient G1 cyclin gene expression.

Discussion

High level expression of the *CLN1* and *CLN2* G1 cyclin genes are required for a yeast cell to pass through START, the commitment point of the cell cycle (Wittenberg and Reed, 2005; Bloom and Cross, 2007). Here, we provide new mechanistic insights into the control of these tightly regulated genes, with implications for control of metazoan cell cycle genes. (1) Nucleosomes are transiently evicted from the *CLN1* and *CLN2* promoters during the cell cycle when the genes are activated, and the FACT chromatin reorganizing complex is required for both nucleosome eviction and transcriptional activation. (2) The Swi6 subunit of the SBF and MBF G1-specific transcription factors recruits FACT to G1-specific promoters. (3) The Rpd3(L) HDAC is also recruited to SBF-dependent promoters through two intermediary proteins, Stb1 and Whi5, that interact with SBF and thus inhibit transcription by recruiting the HDAC. (4) Only Stb1 is required to recruit Rpd3(L) to MBF-dependent promoters, as Whi5 does not interact with MBF. (5) The Cdc28 CDK that drives cell cycle progression is also recruited to SBF-dependent genes by Swi6 and Whi5. (6) FACT and the Cdc28 CDK are both required for viability, but normally temperature-sensitive mutations can be suppressed by mutations in *STB1* and *WHI5*, suggesting that FACT and Cdc28 have important functions in the activation of G1 targets genes. (7) Finally, combining mutations in FACT and SBF results in lethality, but the suppression of this synthetic lethality by a multicopy *CLN2* plasmid, originally isolated as a multicopy suppressor of a *cdc28* temperature-sensitive mutant (Hadwiger *et al*, 1989), shows a critical function for FACT and SBF in activating expression of G1 cyclin genes to regulate the G1/S transition.

The Whi5 inhibitor of G1-specific transcription has been described as analogous to the mammalian Rb transcriptional inhibitor (Schaefer and Breeden, 2004). In mammalian cells, G1-specific promoters are bound by the heterodimeric E2F activator, which is inhibited by Rb. Rb phosphorylation by cyclin D-CDK4/6 at G1/S ends the Rb-E2F interaction and thus allows transcriptional activation (Iaquinta and Lees, 2007; van den Heuvel and Dyson, 2008). In yeast, Whi5 binds to and inhibits the heterodimeric SBF activator, and Whi5 phosphorylation by the Cdc28 CDK results in nuclear export (Costanzo *et al*, 2004; de Bruin *et al*, 2004). Rb inhibits transcriptional activation by recruiting HDACs and we show that the Rpd3(L) HDAC is recruited to the promoters of yeast G1-specific genes. Nuclear entry by Swi6 is regulated within the cell cycle (Sidorova *et al*, 1995), and ChIP experiments show that SBF binds to promoters transiently in the cell cycle. Comparing the binding kinetics at *CLN2* supports the

hypothesis that SBF recruits Rpd3(L) (Figure 1A). However, Rpd3(L) binding decays more rapidly, consistent with the idea that Cdc28 phosphorylation of Whi5 results in loss of both Whi5 and Rpd3(L) from the promoter even while SBF remains bound (Figure 7). The kinetics of Cdc28 recruitment to the *CLN2* promoter (Figure 1A) are consistent with this model.

stb1 and *whi5* mutations have different effects on transcriptional activation of cyclin genes (Figure 2D). RNA levels are decreased in a *stb1* mutant, consistent with Stb1 functioning as a transcriptional activator (de Bruin *et al*, 2008). Interestingly, a *whi5* mutation suppresses the defect in SBF-dependent gene expression caused by *stb1*. One might expect to see increased expression of G1 cyclin genes in the *whi5* single mutant, as *WHI5* is a negative regulator of SBF-dependent genes (Costanzo *et al*, 2004; de Bruin *et al*, 2004). However, the major effect of a *whi5* mutation is seen in assays measuring the size of cells when they pass START (Di Talia *et al*, 2007). Although SBF-dependent genes are activated prematurely in a *whi5* mutant, this would not be seen in our experiments in which the synchrony method allows growth of the bud before cell division. The growth defects caused by FACT mutations were suppressed by *whi5*, whereas a *stb1* mutation showed additive growth defect in combination with a FACT mutation (Figure 6). Additionally, the growth defects of the FACT *stb1* double mutants were suppressed by *whi5*. These growth results are consistent with FACT and Stb1 both stimulating *CLN* gene expression, and Whi5 acting as an inhibitor. We also find that FACT binding to SBF-dependent promoters increases in a *stb1 whi5* mutant, but no change is seen at MBF promoters (Figure 5). Whi5 interacts with the Swi4 subunit of SBF, but not with Mbp1 of MBF (Supplementary Figure S4), and we therefore suggest that Whi5 antagonizes FACT binding. Thus, the defect in *CLN* gene expression caused by the lack of Stb1 could be counteracted at least in part by increased FACT binding, restoring normal expression levels in the *stb1 whi5* double mutant.

FACT binds to the promoters of SBF- and MBF-activated genes transiently during the cell cycle (Figure 4). Interestingly, FACT binding slightly precedes both chromatin disassembly and gene transcription. FACT binding at *POL1* is later in the cell cycle, and chromatin disassembly and *POL1* expression also occur later. A *swi6* mutation eliminating both SBF and MBF prevents FACT recruitment, chromatin disassembly, and gene expression at *CDC21*, and *POL1*, both MBF genes. In contrast, we see a number of differences at *HO* and the two *CLN* genes, although all are activated by SBF. Synchrony experiments show that *HO* expression is largely eliminated by mutations affecting SBF or FACT, but expression of *CLN2* is delayed in these mutants (Supplementary Figure S6). Similarly, although these mutations completely eliminate coactivator binding to *HO* URS2 (Takahata *et al*, 2009), they merely cause a delay in binding of the SAGA coactivator to *CLN2*. How is *CLN2* activated in the absence of SBF? Overexpression of Rme1 increases expression of *CLN2* and also permits growth of normally inviable cells lacking both SBF subunits (Toone *et al*, 1995). The Rme1 DNA-binding protein binds to the *CLN2* promoter *in vitro* and activates through promoter sequences distinct from the SBF-binding sites (Toone *et al*, 1995). We suggest that Rme1 is responsible for the residual *CLN2* activation in strains with SBF or FACT mutations, and that the *HO* promoter lacks Rme1-binding sites. Supporting this, there is an additive

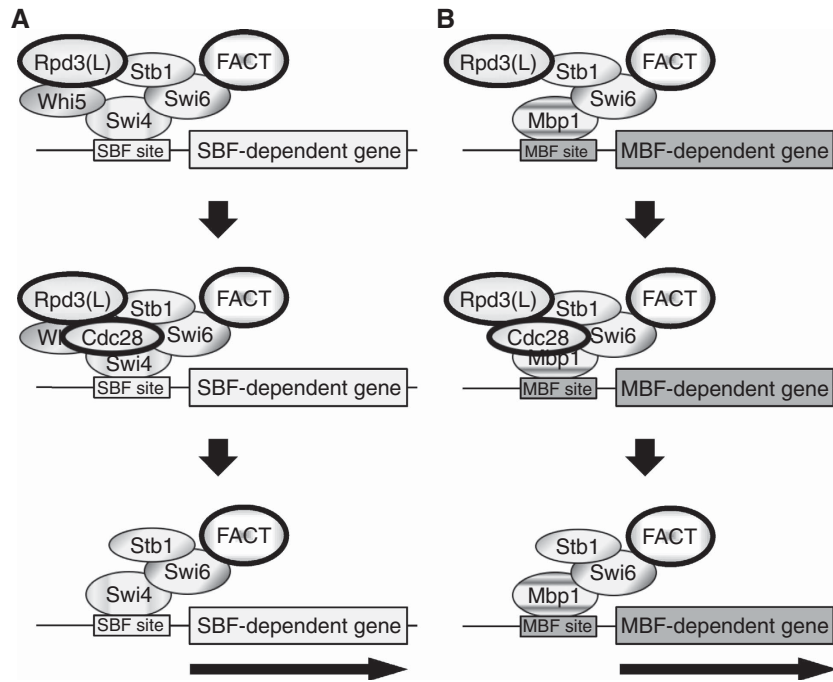


Figure 7 SBF recruits multiple components to promoters. (A) Swi6 enters the nucleus in late M/early G1, interacts with Swi4 to form SBF, and binds to promoters. Whi5 is recruited through interaction with Swi4, and Stb1 and FACT are recruited through interaction with Swi6. Whi5 and Stb1 recruit the Rpd3(L) HDAC. As cells pass START in the cell cycle, the Cdc28 kinase is active and phosphorylates Whi5 and Stb1. Phosphorylated Whi5 leaves the nucleus. We suggest phosphorylated Stb1 remains at the promoter and stimulates gene activation. There appears to be two pulses of FACT binding to *CLN1* and *CLN2* promoters. (B) Mbp1 and Swi6 form the MBF factor, which binds to MBF-dependent promoters. Whi5 is not recruited. Stb1 and FACT are recruited through interaction with Swi6, and Stb1 recruits Rpd3. We suggest that Stb1 phosphorylation by Cdc28 results in disassociation of Rpd3(L). The kinetics of FACT binding is different at distinct MBF-dependent promoters. A full-colour version of this figure is available at *The EMBO Journal* Online.

decrease in *CLN2* expression in *swi6 rme1* and *pob3 rme1* double-mutant strains, but *HO* expression is not affected by a *rme1* mutation (Supplementary Figure S7). Finally, we see two peaks of FACT binding at *CLN1* and *CLN2*, one at 25 min after release and one at 50 min. The two waves of FACT binding could contribute to nucleosome disassembly and reassembly, respectively, but further studies are needed to verify this speculation. It is also surprising that the kinetics of FACT binding to *HO* most closely resemble that of *CDC21*, an MBF-activated gene, instead of *CLN1* and *CLN2*. Although *HO*, *CLN1* and *CLN2* are all SBF-dependent genes, there are important differences, including stringent chromatin repression uniquely seen at the *HO* promoter (ST, YY and DJS, paper in preparation). Overall, these results underscore the way that the same factors can have fundamentally different functions at different promoters.

It is critically important for a cell to tightly regulate expression of genes such as *CLN1* and *CLN2* that control the G1/S transition. These genes are transcriptionally silent, and then rapid expression is stimulated by a positive feedback loop (Skotheim *et al*, 2008). SBF recruits a number of regulatory components to these promoters: Stb1, Whi5, Rpd3(L), FACT and Cdc28 (Figure 7). Chromatin structure at the promoters is probably affected in opposing manner by Rpd3(L) and FACT. We suggest that the Rpd3(L) HDAC produces a repressed state to help keep the gene inactive. Meanwhile, FACT is working to disassemble nucleosomes from the promoter to activate the gene. SBF also recruits the Cdc28 CDK that triggers START through positive feedback, causing nuclear export of Whi5 by phosphorylation.

We suggest that Cdc28 also acts on Stb1 at this time, as the *whi5* mutation causes only a mild reduction in Rpd3(L) binding (Supplementary Figure S1A), whereas Rpd3(L) binding is eliminated in the *stb1 whi5* double mutant (Figure 2A). Stb1 is a target for Cdc28 phosphorylation, and phosphorylated Stb1 no longer interacts with Swi6 (Ho *et al*, 1999; Costanzo *et al*, 2003). ChIP experiments show Stb1 bound at SBF- and MBF-dependent promoters during G1, and the loss of Stb1 binding when transcriptional stops is consistent with phosphorylation of Stb1 eliminating binding to Swi6 (de Bruin *et al*, 2008). Thus, Stb1 and Whi5 both contribute to Rpd3(L) recruitment during early G1, before Cdc28 is activated.

There are significant differences in the regulation of SBF- and MBF-dependent genes. Rpd3(L) is recruited to SBF genes by both Stb1 and Whi5, and Rpd3(L) binding is only lost in the *stb1 whi5* double mutant. Whi5 does not bind to MBF, and only Stb1 is required for Rpd3(L) recruitment to MBF genes. This raises questions as to how Stb1 regulates gene activity. ChIP experiments show Stb1 bound to MBF promoters throughout G1, until transcription terminates (de Bruin *et al*, 2008); this correlates with the time Swi6 is nuclear (Sidorova *et al*, 1995; Geymonat *et al*, 2004). Stb1 is phosphorylated by Cdc28 at START (Ho *et al*, 1999), and this Stb1 modification could release Rpd3(L) from the promoter. We suggest that phosphorylated Stb1 remains bound at the promoter when the gene is transcribed, as a *stb1* mutation reduces activation of SBF and MBF target genes. However, an *in vitro* experiment shows that Stb1 phosphorylation reduces its interaction with Swi6 (Costanzo *et al*, 2003), and thus

further work is needed to understand how Stb1 regulates target genes.

How does loss of Rpd3(L) by the positive feedback loop trigger gene activation? One possibility is that FACT is much less effective at evicting deacetylated nucleosomes from a promoter. Although there is no biochemical data to support this idea, genetic experiments show that FACT defects can be suppressed by an *rdp3* mutation (Formosa *et al*, 2001), and FACT histone acetyltransferase double mutants show synthetic growth defects (Formosa *et al*, 2002). An alternative and nonexclusive regulatory mechanism has Rpd3(L) deacetylating transcription factors at the promoter and thus blocking transcription initiation. In support of this idea, Swi6 interacts both genetically (Macpherson *et al*, 2000) and physically (Sanders *et al*, 2002) with subunits of TFIID, and TAF mutants are defective in the activation of G1/S cyclin genes (Walker *et al*, 1997). Further work is needed to decipher the regulatory mechanisms at these promoters.

Materials and methods

All yeast strains used are listed in Supplementary Table I and are isogenic in the W303 background (Thomas and Rothstein, 1989). Standard genetic methods were used for strain construction (Rothstein, 1991; Sherman, 1991). Plasmid M5056 (YcP:*KanMX::CDC28-HA*) was constructed by inserting the *CDC28-HA* allele from plasmid YcP:*TRP1::CDC28-HA* (Morris *et al*, 2003), kindly provided by Steve Reed, into plasmid pLEJ009 (Jauert *et al*, 2005), provided by David Kirkpatrick. The YcP(*URA3::POB3*) plasmid pJW4 (Schlesinger and Formosa, 2000) was provided by Tim Formosa. Multicopy plasmids were the YEp(*LEU2::CLN1*) (pJH1-38) and YEp(*LEU2::CLN2*) (pJH3-45) (Hadwiger *et al*, 1989), provided by Steve Reed, and YEp(*LEU2::SPT15*) (pSH223) provided by Steve Hahn, and YEp13 (Broach *et al*, 1979) as the empty vector control. Cell cycle synchronization was performed by galactose withdrawal and re-addition with a *GALp::CDC20* strain grown at 25°C in YEP medium containing 2% galactose and 2% raffinose (Bhoite *et al*, 2001). A high degree of synchrony was shown by flow cytometry analysis, budding indices and analysis of cycle-regulated mRNAs (data not shown). In other experiments, cells were grown in YEPD medium (Sherman, 1991).

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ChIPs were performed as described (Bhoite *et al*, 2001; Voth *et al*, 2007) using 9E11 (Abcam) or 4A6 (Upstate) monoclonal antibody to the Myc epitope, monoclonal antibody to the HA epitope (12CA5, University of Utah Bioprocessing Resource), anti-histone H3 (07-690, Upstate), rabbit anti-Spt16 (provided by Tim Formosa), and antibody-coated magnetic beads (Rabbit and Pan Mouse IgG beads, Dynal Biotech). ChIP assays were analysed by real time PCR as described (Eriksson *et al*, 2004). PCR primers are listed in Supplementary Table II. Each ChIP sample was first normalized to an input DNA sample and then to the ChIP signal for a control region on chromosome I. Error bars in ChIP assays reflect the standard deviation of three replicate PCRs. For cell synchrony experiments, the ChIP values were normalized so that the zero time point was 1.0. RT-qPCR was used to measure *HO* mRNA levels as described (Voth *et al*, 2007) using primers listed in Supplementary Table II, except that *RDN25* RNA was used as the internal control. Error bars in RT-qPCR assays reflect the standard deviation of three replicate PCRs. The GST-Swi6 expression plasmid M5435 contains a PCR fragment with the entire SWI6 open reading frame cloned into pGEX4-T1 (Amersham) cleaved with BamHI and XhoI. GST-Swi6 and GST-Swi5 were expressed and purified from *E. coli* as described (Takahata *et al*, 2009). Immunoprecipitations were performed as described earlier (Biswas *et al*, 2008a) using anti-Myc (4A6, Upstate) and anti-Flag (M2, Sigma) antibodies, and blots probed with anti-Myc, anti-Flag, anti-Spt16 and anti-Pob3 (provided by Tim Formosa) antibodies and scanned using a Odyssey Infrared Imaging System (Li-Cor Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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