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# Forkhead proteins control the outcome of transcription factor binding by antiactivation

**Warren P Voth[1], Yaxin Yu[1], Shinya Takahata[1], Kelsi L Kretschmann[1], Jason D Lieb[2,3], Rebecca L Parker[1,4], Brett Milash[1] and David J Stillman[1,*]**

[1]Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT, USA, [2]Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA and [3]Carolina Center for Genome Sciences, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

**Transcription factors with identical DNA-binding specificity often activate different genes *in vivo*. Yeast Ace2 and Swi5 are such activators, with targets we classify as Swi5-only, Ace2-only, or both. We define two unique regulatory modes. Ace2 and Swi5 both bind *in vitro* to Swi5-only genes such as *HO*, but only Swi5 binds and activates *in vivo*. In contrast, Ace2 and Swi5 both bind *in vivo* to Ace2-only genes, such as *CTS1*, but promoter-bound Swi5 fails to activate. We show that activation by Swi5 is prevented by the binding of the Forkhead factors Fkh1 and Fkh2, which recruit the Rpd3(Large) histone deacetylase complex to the *CTS1* promoter. Global analysis shows that all Ace2-only genes are bound by both Ace2 and Swi5, and also by Fkh1/2. Genes normally activated by either Ace2 or Swi5 can be converted to Ace2-only genes by the insertion of Fkh-binding sites. Thus Fkh proteins, which function initially to activate *SWI5* and *ACE2*, subsequently function as Swi5-specific antiactivators.**

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## Introduction

Transcription factors with identical DNA-binding domains can activate different genes *in vivo*, and this constitutes an important unsolved puzzle in gene regulation. Proposed explanatory models include combinatorial control with other DNA-binding factors or subtle differences in binding affinity not detected *in vitro*. Here we present a different paradigm.

Ace2 and Swi5 are cell-cycle-regulated transcription factors in *Saccharomyces cerevisiae* that activate genes expressed in late M and early G1 phase (Simon *et al*, 2001). Ace2 and Swi5 have essentially identical DNA-binding domains and recognize the same sequences *in vitro* (Dohrmann *et al*, 1996), but they activate different genes *in vivo* (Dohrmann *et al*, 1992). For instance, the *HO* gene is only activated by Swi5, while *CTS1* is only activated by Ace2. We show that Fkh1 and Fkh2 can act as selective antiactivators, blocking Swi5, but not Ace2, from activating transcription. Fkh1 and Fkh2 are G2/M phase-specific activators for a set of genes that includes Ace2 and Swi5. Fkh1 and Fkh2 also function as repressors at *CTS1*, but only against Swi5-dependent activation even though Swi5 and Ace2 bind equivalently to the promoter. This specific antiactivation requires recruitment of the Rpd3(Large) histone deacetylase (HDAC) complex. Our global analysis suggests that this mechanism operates *in vivo* at all yeast genes that are bound by these factors. Moreover, we show that Fkh antiactivation is transferable. Promoters that are naturally activated by either Swi5 or Ace2 can be converted to an Ace2-only activation program by insertion of Fkh-binding sites.

*Corresponding author. Department of Pathology, University of Utah, 15 N Medical Drive East, Salt Lake City, UT 84112, USA.
Tel.: +1 801 581 5429; Fax: +1 801 581 4517;
E-mail: david.stillman@utah.edu
[4]Present address: Department of Chemistry, University of Utah, Salt Lake City, UT, USA

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## Results and discussion

### *Genetic identification of CTS1 NRE and Fkh regulatory factors*

We identified a negative regulatory element (NRE) in the *CTS1* promoter which, when deleted or mutated, allowed Swi5 to activate a *CTS1-lacZ* plasmid reporter (Dohrmann *et al*, 1996). This NRE was localized to 66 bps located between −404 and −470 bp upstream of the ATG of *CTS1*, with two Ace2-binding sites located nearby, between −520 and −545 bp (Figure 1A). The NRE represses activation independent of position, orientation, and promoter context (Dohrmann *et al*, 1996). We precisely deleted the minimal NRE region from the genomic *CTS1* promoter, eliminating concerns about effects of a plasmid-based assay or the use of a *lacZ* reporter gene. This *CTS1*(*nre*Δ) allele was introduced into strains with mutations in *ACE2* and *SWI5*, and expression of wild-type *CTS1* and *CTS1*(*nre*Δ) were measured (Figure 1B). The native *CTS1* gene is not expressed in the *ace2* mutant (column 3), but strong expression of *CTS1*(*nre*Δ) is seen even in the absence of the normally required Ace2 activator (column 7). Furthermore, activation of *CTS1*(*nre*Δ) is Swi5-dependent, since expression is lost in the *ace2 swi5 CTS1*(*nre*Δ) strain (column 8). We also constructed a mutant *CTS1(nre-m)* promoter with sequence substitutions throughout the NRE, maintaining the spacing between the Swi5/Ace2-binding sites and the transcription start site, and this mutant promoter showed similar activation by Swi5 (Figure 1B, columns 11–12). Thus, the NRE element in the *CTS1* promoter prevents bound Swi5 from activating *CTS1* transcription. We note that the NRE deletion does not fully restore *CTS1* expression in the absence of Ace2, suggesting that additional repressive mechanisms are still present in the *CTS1*(*nre*Δ) promoter.

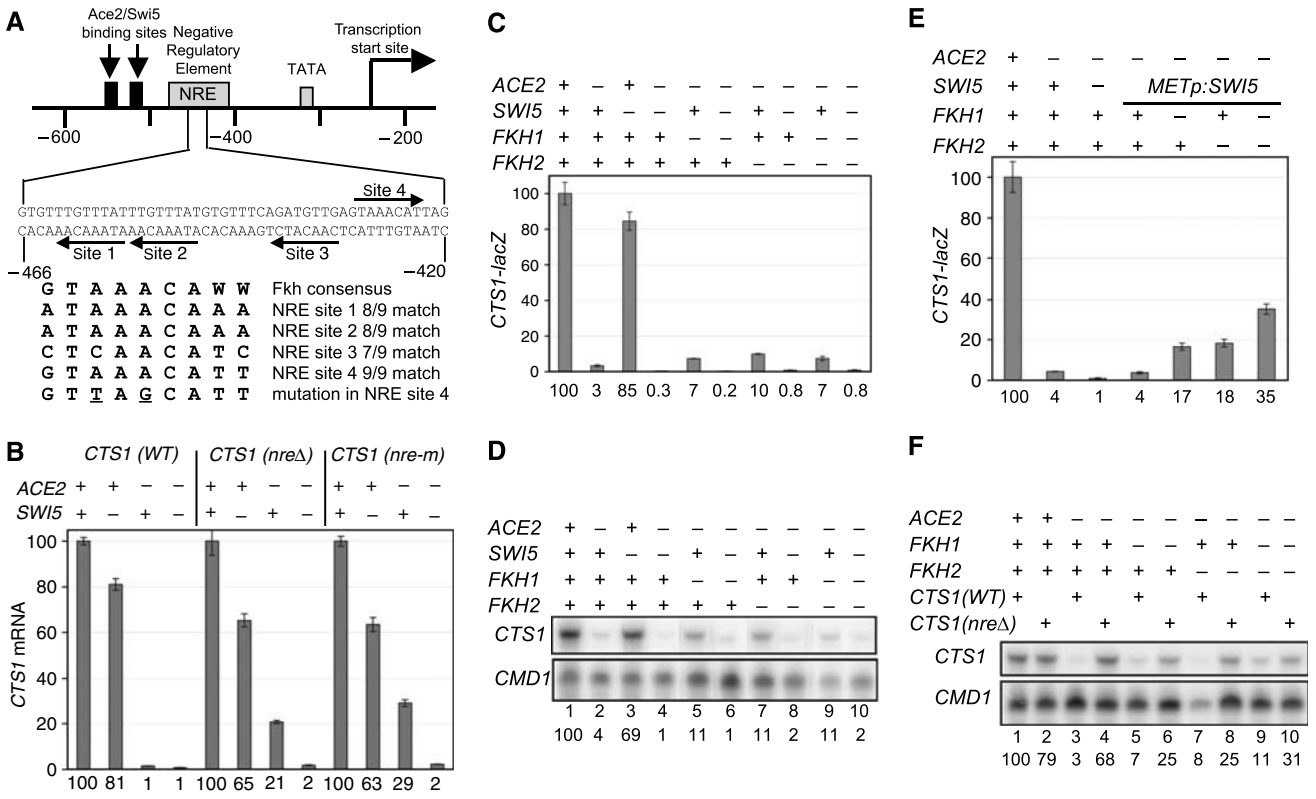



**Figure 1** The NRE in the *CTS1* promoter blocks activation by Swi5. (**A**) Map shows the Ace2/Swi5-binding sites at −546 and −526 from the ATG codon, the negative regulatory element (NRE) defined by deletion analysis as from −470 to −418 (Dohrmann *et al*, 1996), and the TATA element at −313. The transcription start site is at −238 (Dohrmann *et al*, 1992). The four Fkh-binding sites within the NRE are shown. The alignment shows the Fkh consensus site (Zhu *et al*, 2000), the sites from the *CTS1* NRE and the 'TG' mutation in NRE site 4 (Dohrmann *et al*, 1996). (**B**) Swi5 activates *CTS1(nre*Δ*)*. RNAs were used for RT-real-time–PCR assays to measure *CTS1* RNA levels. RNA values are normalized to the *CTS1(WT) ACE2 SWI5* strain (defined as 100), and the error bars show the standard deviation of the triplicate PCR reactions. (**C**) Swi5 weakly activates *CTS1-lacZ* in an *fkh1 fkh2* mutant by β-galactosidase activity from *CTS1-lacZ*. LacZ values are normalized to the *ACE2 SWI5 FKH1 FKH2* strain (defined as 100), and the error bars show the standard deviation of triplicate cultures for lacZ assays. (**D**) Swi5 weakly activates *CTS1* in an *fkh1 fkh2* mutant by S1 nuclease protection assay. (**E**) Swi5 activates *CTS1-lacZ* in an *fkh1 fkh2* mutant when Swi5 is expressed from the Fkh-independent *MET17* promoter. LacZ values are normalized to the *ACE2 SWI5 FKH1 FKH2* strain (defined as 100), and the error bars show the standard deviation of triplicate cultures for lacZ assays. (**F**) Fkh1 and Fkh2 repress *CTS1* via the NRE element by S1 nuclease protection assay.

A genetic screen was carried out to identify mutations in genes that normally prevent Swi5 from activating *CTS1* (Dohrmann *et al*, 1996). An *ace2* strain with an integrated *CTS1-lacZ* reporter was mutagenized and suppressors were identified as blue colonies in the presence of the X-Gal chromogenic substrate. Genetic analysis and complementation cloning for one suppressor mutation, *nce11*, identified plasmids containing the *FKH2* gene. Segregation analysis demonstrated that the *nce11* mutations were allelic with an *fkh2* disruption allele. *FKH2* encodes a member of the winged helix superfamily of DNA-binding transcription factors. Fkh2 and its paralog Fkh1 are redundant activators that bind to the promoters of the *CLB2* group of genes expressed in G2, including *CLB2, SWI5,* and *ACE2* (Hollenhorst *et al*, 2000; Koranda *et al*, 2000; Kumar *et al*, 2000; Pic *et al*, 2000; Zhu *et al*, 2000). Strains with single mutations in *FKH1* or *FKH2* are predominantly normal in cell cycle progression, but *fkh1 fkh2* double mutant strains exhibit strong defects consistent with reduced *CLB2* expression. Although we only obtained an *fkh2* mutant in our initial screen, we included *FKH1* in subsequent analyses, based on its close homology and known functional overlap with *FKH2*.

### *Fkh1 and Fkh2 bind CTS1 and repress via the NRE element*

Strains were constructed to assess the contributions of *FKH1* and *FKH2* in blocking Swi5 activation of *CTS1*. Deletion of either *FKH1* or *FKH2* weakly suppresses the *ace2* defect in *CTS1* expression, allowing Swi5 to activate *CTS1* at 2–3 times the level observed in an *ace2* mutant; similar effects were seen with both an integrated *CTS1-lacZ* reporter (Figure 1C) and the native *CTS1* gene (Figure 1D). Suppression of the *ace2* defect in *CTS1* expression requires Swi5. Thus, mutations in either *fkh1* or *fkh2* have similar effects, allowing Swi5 to inappropriately activate expression of *CTS1*.

Transcription of the *SWI5* gene is normally activated by Fkh1/2 (Hollenhorst *et al*, 2000; Koranda *et al*, 2000; Kumar *et al*, 2000; Pic *et al*, 2000; Zhu *et al*, 2000), so the non-additive suppression of the *ace2* transcriptional defect in the *fkh1 fkh2* double mutant (Figure 1C and D) could be due to decreased *SWI5* expression. To address this problem, a similar set of strains was constructed with *SWI5* expressed from the *MET17* promoter. *MET17* expression under noninducing conditions is Fkh-independent and occurs at S/G2 (Spellman *et al*, 1998), when *SWI5* is expressed. Swi5 produced from

this *METp*::*SWI5* construct retains the post-translational signals for regulated nuclear localization and degradation within the cell cycle. Immunoblot quantitation shows levels of Swi5 expressed from the *MET17* promoter are less than two-fold above native Swi5 (data not shown). Using this *METp*::*SWI5* allele, *CTS1* expression in the absence of Ace2 increased four- to five-fold with either a *fkh1* or *fkh2* single mutation, and the *ace2 fkh1 fkh2* mutant showed *CTS1* expression levels about nine-fold over that of *ace2* alone (Figure 1E). The additive increase in Swi5-dependent *CTS1* expression in the *fkh1 fkh2* double mutant indicates that the Fkh1 and Fkh2 factors are partially redundant for inhibiting *CTS1* activation by Swi5, but both are required for full repression. In contrast, we do not see additivity in the *fkh1 fkh2* double mutant when *SWI5* is expressed from its native Fkh1/2-dependent promoter (Figure 1C and D). The additive effect in the *fkh1 fkh2* double mutant when *SWI5* is expressed from the Fkh-independent *MET17* promoter (Figure 1E) indicates that the Fkh proteins are redundant. Additionally, combining the *fkh1* and *fkh2* mutations with the NRE deletion shows only a minor increase in suppression relative to the effect of the NRE deletion alone (Figure 1F). This comparative lack of additivity is consistent with Fkh1/2 acting through the NRE element at *CTS1*.

The *CTS1* NRE region contains four matches to the consensus Fkh1-binding site (Zhu *et al*, 2000), with one site being a perfect match (Figure 1A). We previously identified a two-nucleotide substitution in the NRE region that allows Swi5 activation (Dohrmann *et al*, 1996), and these changes are within the perfect Fkh consensus site. Chromatin immunoprecipitation (ChIP) shows that Fkh1 and Fkh2 both bind to the *CTS1* and *CLB2* promoters, but no binding was seen to the *CTS1*(*nre*Δ) allele (Figure 2A and B) demonstrating that Fkh1 and Fkh2 bind specifically to the NRE region of the *CTS1* promoter.

### *Binding of the Rpd3(Large) complex to the NRE element*

Recent work has suggested that Fkh2 recruits the Isw2 remodeling factor to promoters, and that Isw2 contributes to transcriptional repression (Sherriff *et al*, 2007). To investigate whether Isw2, or its paralog Isw1, were involved in blocking Swi5 activation at *CTS1*, we constructed *ace2 isw2*, *ace2 isw1*, and *ace2 isw1 isw2* strains. The substantial reduction in *CTS1* mRNA levels caused by an *ace2* mutation is not suppressed by *isw1* or *isw2* mutations (data not shown), and we conclude that these chromatin regulators do not repress at *CTS1*. We next examined *sin3* and *rpd3* mutations, as Sin3 was identified as interacting with Fkh1 and Fkh2 in a global protein interaction screen (Ho *et al*, 2002), and Rpd3 is associated with Sin3 as components of HDAC complexes

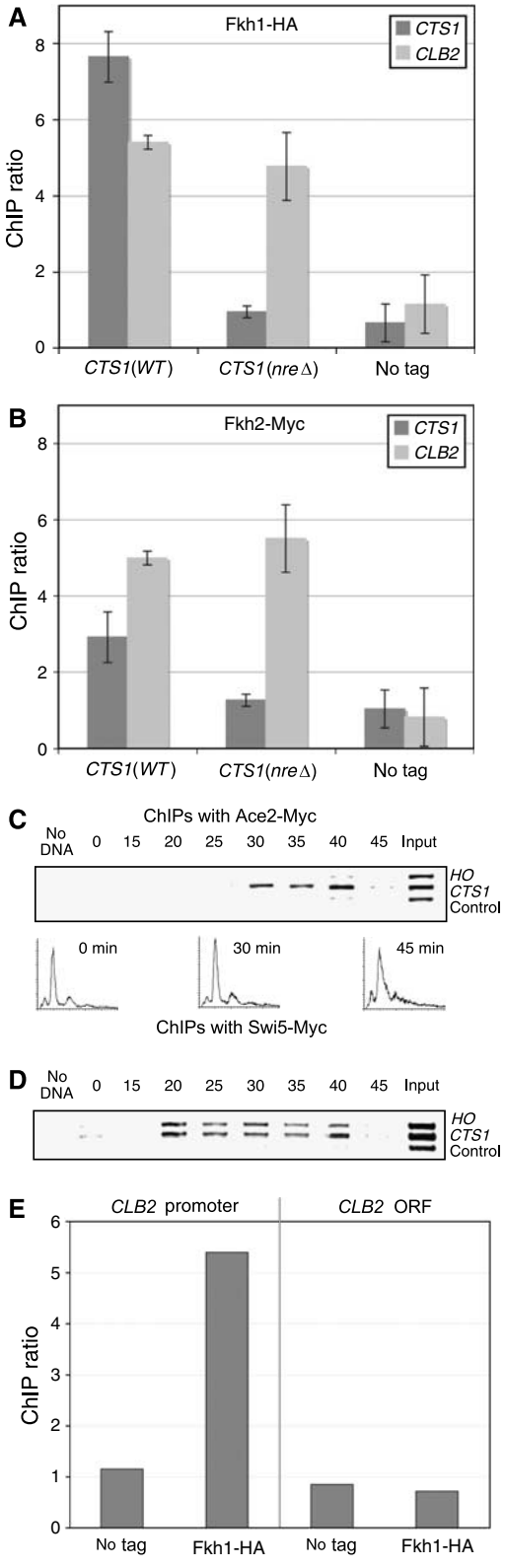



**Figure 2** Fkh1 and Fkh2 bind to the *CTS1* NRE. (**A**) ChIP assay shows Fkh1-HA binding to *CTS1* and *CLB2*. Deletion of the NRE eliminates Fkh1 binding. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**B**) ChIP assay shows Fkh2-Myc binding to *CTS1* and *CLB2*. Deletion of the NRE eliminates Fkh2 binding. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**C**) ChIP assays from synchronized Ace2-Myc *GAL-CDC20* cells show Ace2 binding to *CTS1* but not *HO*. Flow cytometry profiles show the quality of the cell cycle synchrony. The vast majority of these haploid cells have an apparent 2C DNA content even at later time points, as cells release and progress into G1 without cell separation, which is only beginning at the 45 min time point. The strain has an *swi5* mutation, and the modest defect in cell separation in an *swi5* mutant (Dohrmann *et al*, 1992) results also in a small number of cells with apparent 4C DNA content. Progression from mitotic arrest through G1 and into S phase was also confirmed by synchronous bud formation at later time points (data not shown). (**D**) ChIP assays from synchronized Swi5-Myc *GAL-CDC20* cells show Swi5 binding to *HO* and *CTS1*. (**E**) ChIP assay shows *Fkh1-HA* from log phase cells binding to the *CLB2* promoter but not the *CLB2* ORF.

that repress transcription (Kurdistani and Grunstein, 2003). *CTS1* mRNA levels are higher in *ace2 sin3* and *ace2 rpd3* strains than in an *ace2* single mutant, indicating that Sin3/Rpd3 is involved in *CTS1* repression (Figure 3A and B).

At least two HDAC complexes contain Rpd3 (Carrozza *et al*, 2005; Keogh *et al*, 2005). The Rpd3(Large) complex is present primarily at promoters while Rpd3(Small) acts primarily at transcribed open reading frames (ORFs). While both complexes contain Sin3 and Rpd3, there are unique subunits such as Sds3 present only in Rpd3(Large), and Rco1 present only in Rpd3(Small). An *sds3* mutation allows *CTS1* expression in the absence of Ace2, while an *rco1* mutation does not (Figure 3A and C), and thus Rpd3(Large) represses *CTS1*. The *CTS1*(*nre*Δ) allele also allows *CTS1* activation by Swi5, but there is no additive effect from combining this promoter mutation with either *sin3* or *sds3* in the *ace2 SWI5* strains. This lack of additivity suggests that Rpd3(Large) represses *CTS1* expression via the NRE element. As seen for the NRE deletion, neither *sin3* nor *sds3* mutations fully restore *CTS1* expression in the absence of Ace2, suggesting additional mechanisms of repression. Finally, while *sin3* and *sds3* mutations allow *CTS1* expression in the absence of Ace2, *CTS1* expression is largely lost in the *ace2 swi5* strains, demonstrating that Swi5 is responsible for *CTS1* transcription in the absence of Ace2.

ChIP experiments were performed to examine binding of Sin3/Rpd3 complexes to the *CTS1* promoter. Sin3, Rpd3, and Sds3 all bind to *CTS1*, indicating binding by Rpd3(Large); in contrast, Rco1, the Rpd3(Small)-specific subunit, does not bind (Figure 3D). We next compared binding of Rpd3(Large) to the wild-type *CTS1* promoter and the *CTS1*(*nre*Δ) promoter deletion. Deleting the NRE element results in a substantial reduction of binding of Sin3 (Figure 3E) and Sds3 (Figure 3F), demonstrating that the NRE is required for recruiting Rpd3 (Large) to *CTS1*. As a control, we measured binding to the *INO1* promoter, known to bind Sin3/Rpd3 (Kadosh and Struhl, 1997). Binding to *INO1* was unaffected in the *CTS1*(*nre*Δ) strain, demonstrating specificity. Interestingly, Rpd3(Large) binds to the *CLB2* promoter as well, which is also bound by Fkh1 and Fkh2. An *ace2* mutation does not affect Rpd3(Large) binding to *CTS1* (data not shown). We next examined histone acetylation directly at *CTS1* in wild-type and *rpd3* strains. The *rpd3* mutation results in decreased histone H3 at *CTS1*, and increased levels of acetylated H3 and acetylated H4 (Figure 4A). Normalizing the acetylated histone ChIP signals to the levels of H3 at the promoter shows that the *rpd3* mutation results in a marked increase in histone acetylation at the *CTS1* promoter (Figure 4B). We also determined whether Rpd3(Large) binding to promoters was dependent upon Fkh proteins, measuring Sin3-HA binding in wild-type, *fkh1* and *fkh2* single mutants, and in the *fkh1 fkh2* double mutant (Figure 4C). The Sin3 ChIP signal at the *CLB2* and *CTS1* promoters is abolished in the *fkh1 fkh2* double mutant, but largely unaffected in the *fkh1* or *fkh2* single mutants. We conclude that either Fkh1 or Fkh2 is competent to recruit Rpd3(Large) to the *CLB2* and *CTS1* promoters. In contrast, Rpd3(L) binding to the *INO1* promoter is unaffected in the *fkh1 fkh2* double mutant. In summary, these experiments demonstrate that the Rpd3(Large) HDAC complex is recruited to the *CTS1* promoter by Fkh1 and Fkh2, Rpd3(Large) and functions to specifically reduce *CTS1* activation by Swi5.

### *Different in vivo DNA binding by Ace2 and Swi5*

Although *in vitro* binding experiments show that Ace2 and Swi5 recognize the same sequences in promoters, they can activate transcription of different genes *in vivo* (Dohrmann *et al*, 1996). Ace2, but not Swi5, activates *CTS1* expression, and Swi5, but not Ace2, activates *HO*. Ace2 and Swi5 are cell-cycle-regulated transcription factors, and cells with a *GAL*::*CDC20* allele were synchronized within the cell cycle by removing galactose to arrest in mitosis, followed by re-addition of galactose (Bhoite *et al*, 2001). Flow cytometry and analysis of cycle-regulated mRNAs show a high degree of synchrony (Figure 2C and data not shown). ChIP experiments were performed with synchronized cells showing Swi5-Myc binding to the *HO* and *CTS1* promoters (Figure 2D). Swi5-Myc binds to both *HO* and *CTS1*, although it does not activate *CTS1*. Thus, the NRE in *CTS1* does not prevent Swi5 from binding, but acts to prevent promoter-bound Swi5 from activating transcription.

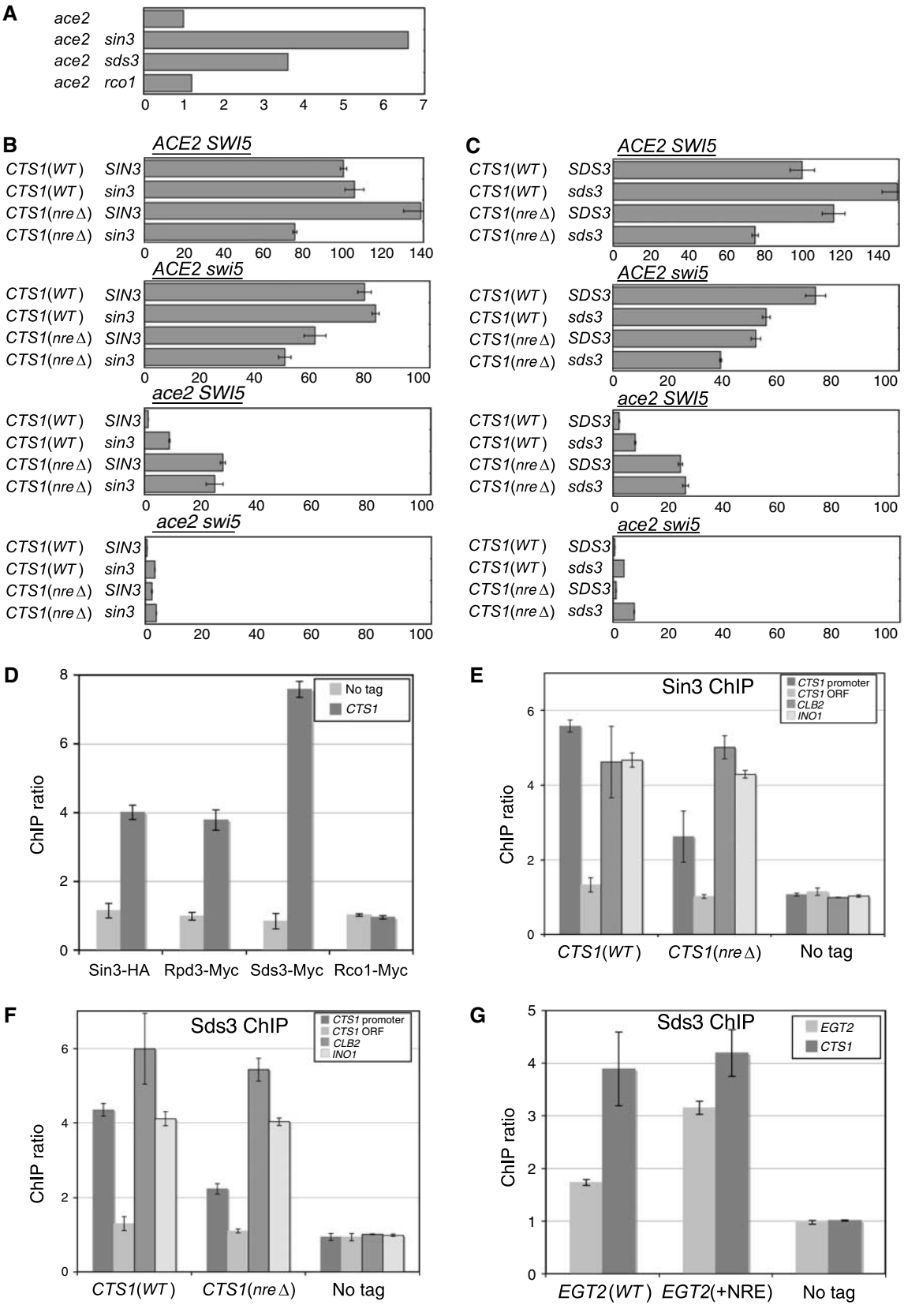
ChIP with Ace2-Myc shows binding to *CTS1* but not to *HO* (Figure 2C); Ace2 does not activate *HO* although it binds *in vitro* (Dohrmann *et al*, 1996). Swi5 does not block Ace2 from binding to *HO*, as no binding of Ace2 to *HO* is seen in either an *SWI5* or an *swi5* strain. Thus, there are distinct mechanisms of promoter restriction for Swi5 and Ace2 at *HO* and *CTS1*. Swi5 binds to *CTS1*, but does not activate transcription. In contrast, Ace2 is unable to activate *HO* transcription simply because it does not bind to the *HO* promoter. During the cell cycle, Swi5 binds DNA before Ace2 (Figure 2C and D), and Swi5 enters the nucleus before Ace2 (Sbia *et al*, 2007). Work is in progress to understand why Ace2 is unable to bind to and activate expression of the *HO* gene, although Ace2 binds well to *HO in vitro*.

### *Genome-wide analysis of regulation and binding by Swi5 and Ace2*

To determine whether these distinct promoter restriction mechanisms are general for all Ace2 and/or Swi5 targets throughout the genome, global expression, chromatin binding, and binding motif correlations were undertaken. First, genome-wide experiments were performed to determine whether these Ace2- and Swi5-binding patterns are seen at other promoters. Expression microarrays were used to define their targets of regulation and revealed that the RNA levels for 66 genes was reduced more than two-fold in either the *ace2* single mutant or the *swi5* single mutant or the *ace2 swi5* double mutant (Supplementary data). To more stringently identify genes directly regulated by Ace2 and Swi5, we eliminated genes that are not cell cycle regulated (Cho *et al*, 1998; Spellman *et al*, 1998; Pramila *et al*, 2006). Among the 23 remaining genes, eight were activated only by Ace2, six by Swi5 only, and nine required either Ace2 or Swi5 for activation (Figure 5A). Expression of *CDC6* and *PCL2* was reduced less than two-fold, but these genes were included in our analysis as their expression was previously shown to be activated by Ace2 or Swi5 (Piatti *et al*, 1995; Aerne *et al*, 1998; McBride *et al*, 1999; Doolin *et al*, 2001). RT–PCR experiments confirm the dependence of these genes on Ace2 and/or Swi5 (Supplementary Figure S1).

To determine Ace2 and Swi5 genomic-binding locations, Ace2-Myc and Swi5-Myc strains were synchronized at M with *GAL*::*CDC20*, and samples taken at timed intervals after the release were processed for ChIP and hybridized

to whole-genome DNA microarrays. The complete experiment was performed three times (Supplementary data). Some promoters were bound by both Swi5 and Ace2, and some by Swi5 only; but none of the selected promoters were bound by Ace2 only (Figure 5D). The analysis revealed that Ace2 and Swi5 both bind to all genes activated only by Ace2, but only Swi5 binds to genes activated only by Swi5. This is the same pattern observed for *CTS1* and *HO* (Figure 2C and D). Cells synchronized with *GAL*::*CDC20* and ChIPs quantitated with real-time



PCR confirm the ChIP–chip results for selected representative genes (Figure 6A).

### *Upstream Fkh sites define genes that are activated only by Ace2*

At *CTS1*, Fkh binding is required to prevent Swi5 from activating transcription. To assess whether Fkh-binding sites are present at other genes activated by Ace2 but not by Swi5 (Ace2-only genes), we analyzed data from the expression microarray and ChIP–chip experiments (Figure 5A and D) in relation to the genome-wide distribution of DNA sequence motifs bound by Ace2/Swi5 and/or Fkh1/2 (MacIsaac *et al*, 2006) (Figure 5B). Ace2-only genes contain both Ace2/Swi5 and Fkh sites (red), while Swi5-only genes

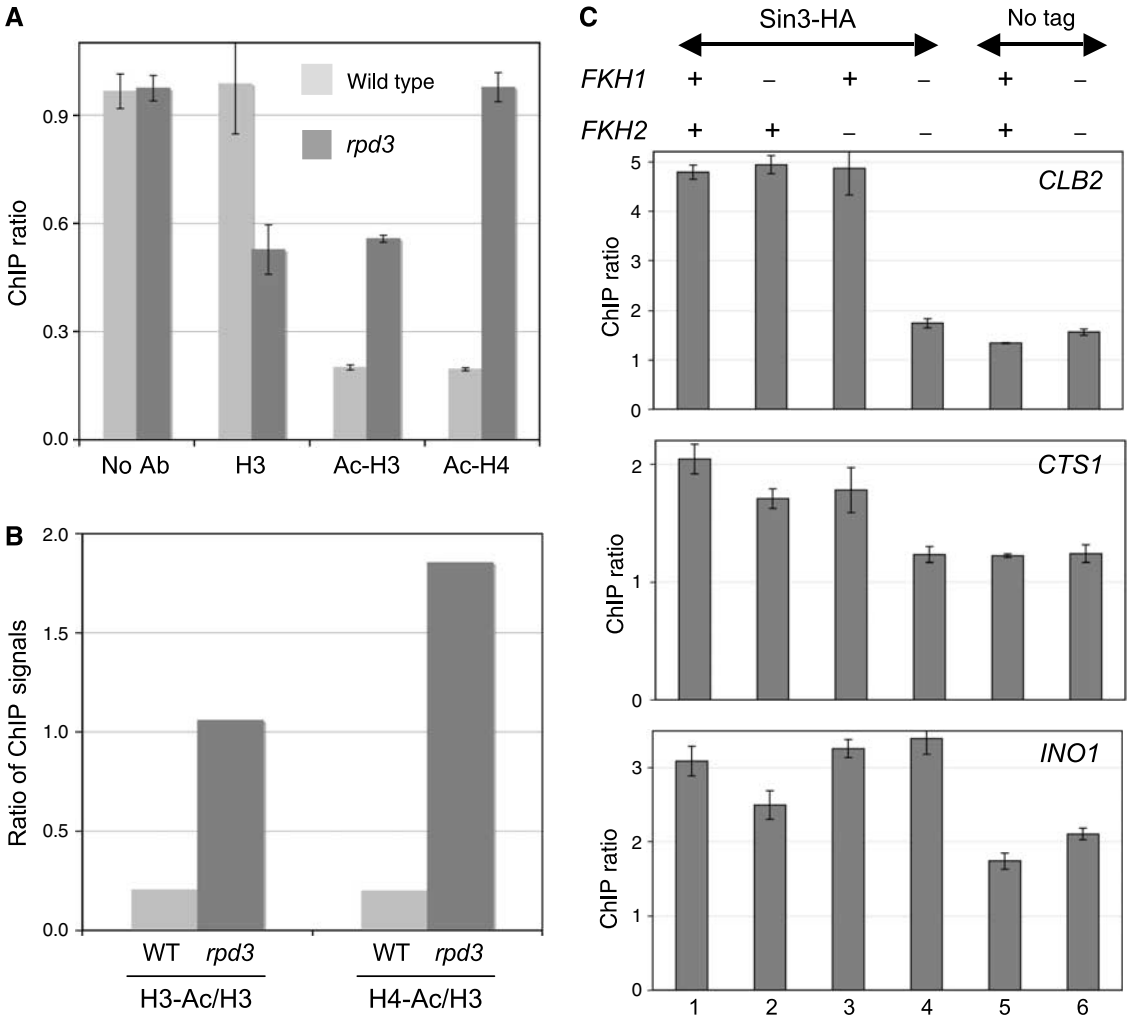



**Figure 4** *rpd3* affects histone acetylation and *fkh* mutation affect Rpd3(L) binding to *CTS1*. (**A**) Lysates from wild-type or rpd3 mutant cells were immunoprecipitated with either no antibody, anti-H3, anti-H3(K14-Ac), or anti-H4(-Ac). PCR reactions were performed with CTS1 and control primers, and the ratio of these ChIP values were normalized to ratios for PCR reactions with the two primer sets using input DNA. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**B**) Using the data in part (A), the ChIP ratios for H3(K14-Ac) and H4(-Ac) were divided by the ChIP ratios for histone H3. (**C**) ChIP assays of Sin3-HA binding in wild-type, *fkh1*, *fkh2*, and *fkh1 fkh2* strains show that either Fkh1 or Fkh2 can recruit Sin3-HA to *CLB2* and *CTS1*, while *fkh* mutations do not affect Sin3-HA binding to the *INO1* promoter.

**Figure 3** Rpd3(Large) regulates *CTS1* via the NRE. (**A**) RT–PCR assays show that *sin3* and *sds3* mutations allow *CTS1* expression despite an *ace2* mutation, while *rco1* does not suppress. RNA values are normalized to the *ace2* mutant (defined as 1); *CTS1* RNA in the *ace2* mutant is 2% of the *ACE2+* strain. (**B**) RT–PCR assays show that *CTS1* can be activated by Swi5 in an *sin3* mutant, and that the effects of an *sin3* mutation and deletion of the NRE promoter element are not additive. RNA values are normalized to the *CTS1(WT) ACE2 SWI5 SIN3* strain (defined as 100), and the error bars show the standard deviation of the triplicate PCR reactions. (**C**) RT–PCR assays show that *CTS1* can be activated by Swi5 in an *sds3* mutant, and that the effects of an *sds3* mutation and deletion of the NRE promoter element are not additive. RNA values are normalized to the *CTS1(WT) ACE2 SWI5 SIN3* strain (defined as 100), and the error bars show the standard deviation of the triplicate PCR reactions. (**D**) The Sin3-HA, Rpd3-Myc, and Sds3-Myc components of Rpd3(Large) bind to *CTS1* in a ChIP assay, while the Rco1-Myc component of Rpd3(Small) does not. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**E**) ChIP assay shows Sin3-HA binding to *CTS1*, *CLB2*, and *INO1*. Deletion of the NRE reduces Sin3 binding. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**F**) ChIP assay shows Sds3-HA binding to *CTS1*, *CLB2*, and *INO1*. Deletion of the NRE reduces Sds3 binding. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate. (**G**) ChIP assay shows Sds3-HA binding to *EGT2* and *CTS1*. Insertion of the NRE element from *CTS1* into the *EGT2*(+NRE) promoter increases Sds3 binding. Error bars show the standard deviation of the ChIP PCR reactions performed in triplicate.

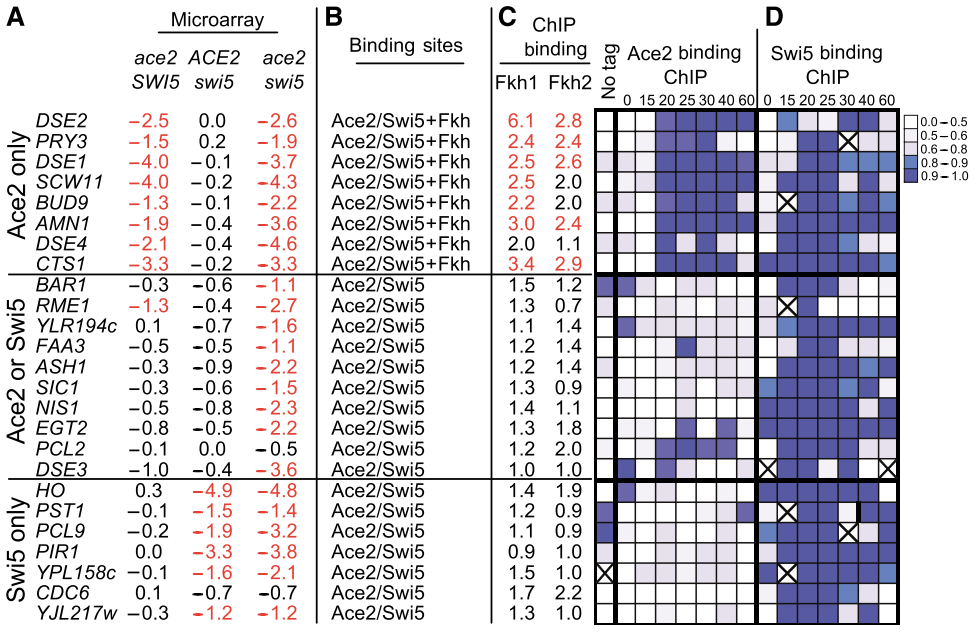



**Figure 5** Expression and promoter occupancy of Ace2- and Swi5-dependent genes. (**A**) Relative RNA levels in *ace2*, *swi5*, and *ace2 swi5* strains as a $\log_2$ ratio (mutant/WT). RNA levels with greater than a two-fold reduction are shown in red. (**B**) The presence of binding sites for Ace2/Swi5 or Fkh1/2 in promoters, from MacIsaac *et al* (2006). (**C**) The degree of Fkh1 or Fkh2 binding by ChIP–chip are the binding ratios from the data supporting Simon *et al* (2001), with binding levels greater than two standard deviations from the mean shown in red. (**D**) Ace2 and Swi5 binding as a heat map showing the results for an untagged control strain, and for the Ace2-Myc and Swi5-Myc strains at the *CDC20* arrest ($t = 0$) and time points after release. The heatmap represents the results of 33 microarray experiments (Materials and methods) with the number of repeats for each time point as shown in Supplementary Table 3. Percentile ranks were calculated for each experiment and then averaged for each time point to generate the values shown. A color scale represents the degree of binding expressed as percentile ranks of ChIP enrichment.

contain Ace2/Swi5 sites but lack Fkh sites (yellow) (Figure 7A). Examining genes whose expression is reduced in an *ace2* mutant, we find that both Ace2 (Figure 7B) and Swi5 (Figure 7C) bind, and all of these genes contain Fkh-binding sites. In contrast, Swi5 (Figure 7D) but not Ace2 (Figure 7E) binds to Swi5-only genes, and none of these contain Fkh sites. The Ace2-only genes all contain multiple Fkh sites, but there is no common pattern of binding site spacing or orientation in the three classes of promoters (Figure 6B). ChIP–chip experiments (Simon *et al*, 2001) show Fkh1 and Fkh2 bind to the Ace2-only genes, but not to either class of gene activated by Swi5 (Figure 5C).

### *Introduction of Fkh sites into promoters reduces activation by Swi5 but not Ace2*

To test whether Fkh sites are sufficient to reduce activation by Swi5, we inserted the *CTS1* NRE, containing Fkh-binding sites into the native promoters of the *EGT2* and *SIC1* genes. *EGT2* and *SIC1* are activated by both Ace2 and Swi5 (Knapp *et al*, 1996; Kovacech *et al*, 1996; Toyn *et al*, 1997), and are not normally bound by Fkh1 or Fkh2 (Simon *et al*, 2001). Expression of *EGT2* and *SIC1* is modestly reduced in *ace2* or *swi5* single mutants, but more substantially reduced in the *ace2 swi5* double mutant (Figure 8). In the *ace2 swi5* strain, *EGT2* levels are reduced to undetectable levels, while *SIC1* mRNA is reduced to 30% of wild type. Presumably, there are other basal activators of *SIC1* expression in addition to Ace2 and Swi5. In contrast, *CTS1* mRNA is absent in the *ace2 SWI5* strain.

We made two types of insertions into the *EGT2* and *SIC1* promoters. Both were made at the endogenous chromosomal locations with no loss of native sequences. The *EGT2*(+NRE) and *SIC1*(+NRE) promoters have a region from the *CTS1* promoter with the entire NRE element inserted, while the *EGT2*(+NRE-m) and *SIC1*(+NRE-m) promoters contain a similar sized fragment from *CTS1* except for sequence substitutions throughout the four Fkh sites (see Figure 1). Expression of *EGT2*(+NRE) and *SIC1*(+NRE) is significantly reduced in the *ace2* strain, where Swi5 is the remaining major activator (Figure 8). Expression of *SIC1*(+NRE) is reduced to nearly the level seen in the *ace2 swi5* strain, providing additional evidence that the NRE blocks activation by Swi5 (Figure 8).

Data from *EGT2*(+NRE) suggests that the NRE is only partially able to block Swi5 activation at *EGT2*. This may be due to the number and dispersed locations of Swi5/Ace2-binding motifs throughout the promoter (see Figure 6B). For both promoters, however, the NRE reproducibly confers at least a two-fold reduction only in Swi5-dependent activation, with no effect on Ace2-dependent activation (Figure 8). This results in an approximately three-fold bias for Ace2 relative to Swi5 for these promoters, which are normally equivalently sensitive to either activator. The *EGT2*(+NRE-m) and *SIC1*(+NRE-m) promoters with a *CTS1* promoter fragment lacking the Fkh sites are expressed at levels similar to that of wild-type promoters, indicating that the observed effects are not due to alterations in spacing or architecture within these promoters. These experiments show that Fkh sites are sufficient to prevent Swi5 from activating specific target genes.

We also examined Sds3 binding to the *EGT2*(+NRE) promoter. As shown in Figure 3G, there is increased Sds3 binding to *EGT2*(+NRE) compared to wild-type EGT2. This

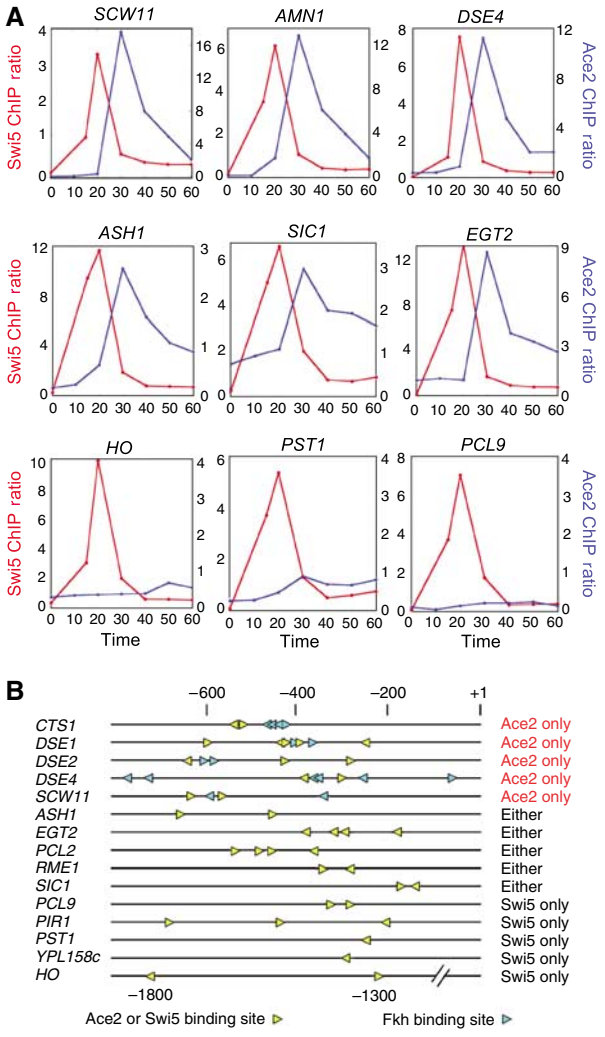



**Figure 6** Binding of Swi5 and Ace2 to target genes. (**A**) Ace2-Myc and Swi5-Myc binding in synchronized *GAL-CDC20* cells were measured by real-time PCR after ChIP. ChIP-binding ratios are expressed as a ratio to an internal control. (**B**) Diagrams show the positions of Ace2- or Swi5-binding sites (yellow arrows) or Fkh-binding sites (blue arrows) on genes characterized as (1) activated by Ace2 only, (2) activated by either Ace2 or Swi5, or (3) activated either by Swi5 only.

demonstrates that the NRE element with Fkh-binding sites is sufficient to recruit the Rpd3(Large) complex to promoters.

### *DNA binding by Fkh1 and Fkh2 varies during the cell cycle but this is unlikely to be required for the antiactivation specificity*

Fkh1 and Fkh2 binding at *CLB2* group promoters activates transcription in G2, yet binding at Ace2-activated targets results in selective repression in G2/M. Additionally, binding of Swi5 and Ace2 to their G1 targets occurs at different times, with Swi5 preceding Ace2. Thus, we hypothesized that differences in Fkh1 and Fkh2 occupancy at *CTS1* and *CLB2* with respect to the cell cycle might determine their roles as either activators versus selective antiactivators. We therefore directly analyzed the Fkh1 and Fkh2 proteins during the cell cycle by ChIP and by western immunoblotting (Supplementary Figure S2). During the cell cycle, ChIP experiments show significant variation in binding to *CTS1* and *CLB2* over time, with maxima during the M and early G1 period. Additionally, western blots show subtle differences in abundance and mobility of Fkh proteins during the cell cycle. However, the cell cycle times of these changes do not explain why Fkh1 and Fkh2 differently repress and activate *CTS1* and *CLB2*. Thus, it appears unlikely that the mechanism of Swi5-specific antiactivation involves restricting Fkh occupancy of *CTS1* to a limited period of the cell cycle.

Activation of *CLB2* involves Fkh2 recruiting the Ndd1 activator at certain cell cycle times (Koranda *et al*, 2000). At other times of the cell cycle, Fkh1 and Fkh2, in the absence of Ndd1, actually repress *CLB2* transcription (Hollenhorst *et al*, 2000), an idea supported by the observation that *fkh1* mutations suppress the lethality caused by deletion of the *NDD1* gene (Koranda *et al*, 2000). *CLB2* activation during G2 requires the presence of Mcm1 at the promoter (Althoefer *et al*, 1995a). At *CTS1*, in the absence of Mcm1 interaction, but where there are multiple Fkh-binding sites, Fkh1 and Fkh2 binding may continuously reinforce each other, resulting in a sharp peak of Fkh2 binding to *CTS1* at G2/M and a subsequent Fkh1 peak in M/G1, resulting in sufficient occupancy by both factors for full repression. Thus, promoter context and neighboring DNA-binding proteins may determine the activity of Fkh1 and Fkh2.

### *Fkh proteins do not bind to the CLB2 ORF*

Because both Ace2 and Swi5 are equivalently able to program Pol II transcription in Fkh-independent contexts, we hypothesized that the mechanism of discrimination may involve *FKH*-dependent differences in the ability of Ace2 and Swi5 to program a step subsequent to Pol II recruitment. Morillon *et al* (2003) reported that Fkh1, in addition to binding to the *CLB2* promoter, also binds to the *CLB2*-coding region. The authors postulated that Fkh proteins influence transcriptional elongation and termination, and regulate phosphorylation of the Pol II CTD. *CLB2* is cell cycle regulated, expressed soon after the release during G2. One prediction based on Morillon *et al* (2003) is that the Fkh proteins would be bound to the promoter before expression, and then appear to travel down the ORF after transcription begins. A variation would be for Fkh1 to be bound to the *CLB2* ORF throughout the cell cycle, irrespective of whether the gene is transcribed or not.

To test these models, we examined the kinetics of Fkh1 binding to this ORF, using chromatin extracts from synchronized cells expressing Fkh1-HA. However, our ChIP assays provided no evidence for Fkh1 binding to the *CLB2* ORF at a level above background during the cell cycle (Supplementary Figure S3A). We also looked for Fkh1 binding to the *CLB2* ORF in log phase cells, as nonsynchronized cells had been used by Morillon *et al* (2003). Once again, we did not see any Fkh1 binding to the *CLB2* ORF, although we did see strong binding to the *CLB2* promoter (Figure 2E) in both sets of samples, as well as strongly periodic binding to the *CTS1* promoter.

Based on the suggestion that Fkh proteins regulate pol II CTD phosphorylation (Morillon *et al*, 2003), we considered a model where Swi5 binding leads to pol II recruitment to *CTS1*, but the Fkh proteins prevent pol II from initiating transcription. However, ChIP shows equivalent pol II binding to *CTS1* in WT versus *swi5* strains, and pol II binding is reduced to equivalent levels in *ace2* and *ace2 swi5* strains (Supplementary Figure S3B). This result rules out a model

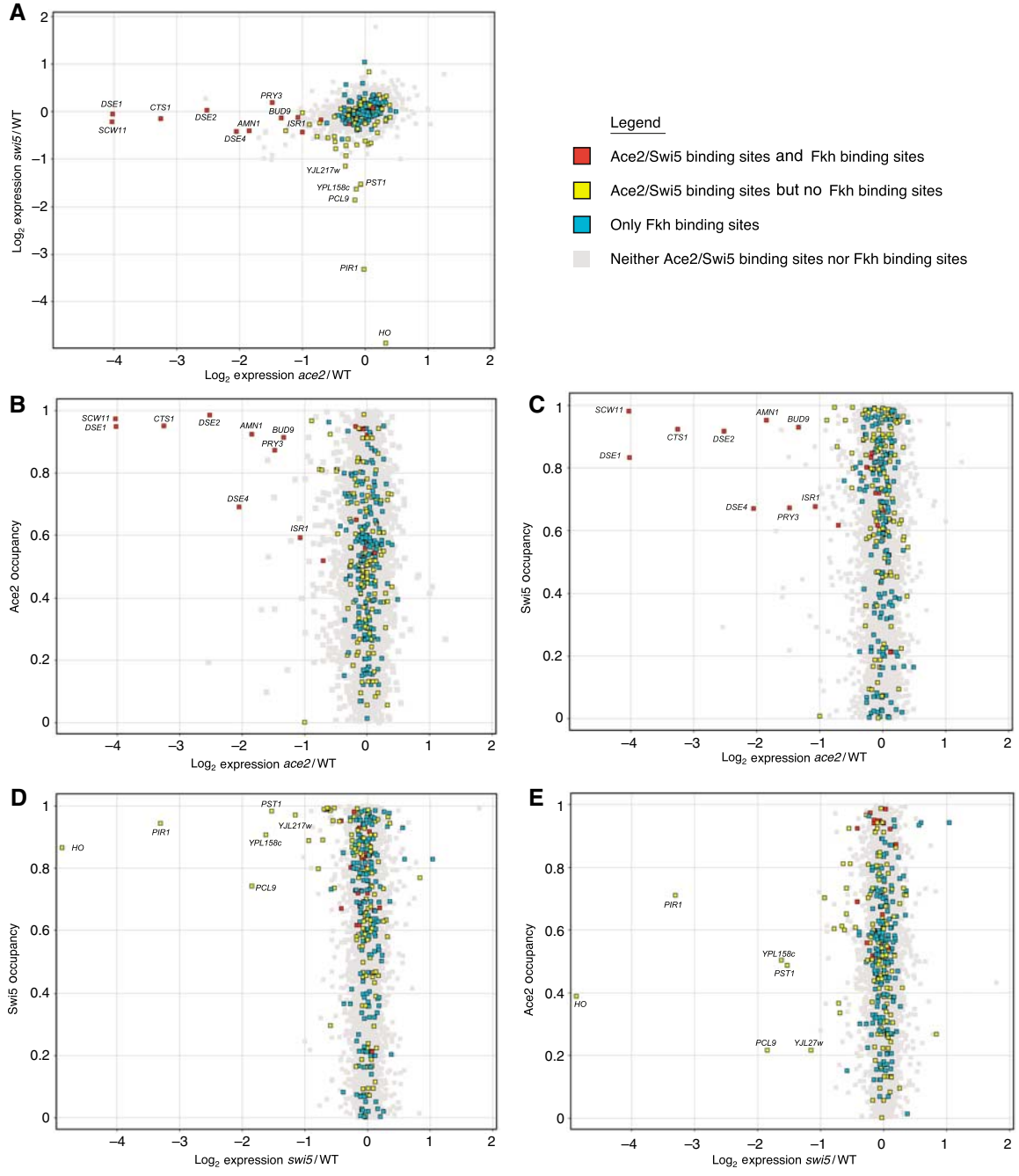



**Figure 7** Genome-wide analysis of expression, binding sites, and factor binding. Promoters with Ace2/Swi5-binding sites and Fkh-binding sites are red, those with Ace2/Swi5 but no Fkh sites are yellow, those with only Fkh sites are blue, and those with neither site are gray. (**A**) $\log_2$(*ace2*/WT) plotted versus $\log_2$(*swi5*/WT) microarray data, showing Ace2-only genes do contain Fkh sites and Swi5-only genes do not. (**B**) $\log_2$(*ace2*/WT) microarray data plotted versus Ace2 ChIP–chip occupancy data, showing Ace2 binding to Ace2-only genes. (**C**) $\log_2$(*ace2*/WT) microarray data plotted versus Swi5 ChIP–chip occupancy data, showing Swi5 binding to Ace2-only genes. (**D**) $\log_2$(*swi5*/WT) microarray data plotted versus Swi5 ChIP–chip occupancy data, showing Swi5 binding to Swi5-only genes. (**E**) $\log_2$(*swi5*/WT) microarray data plotted versus Ace2 ChIP–chip occupancy data, showing Ace2 does not bind to Swi5-only genes.

where Swi5 recruits pol II to *CTS1*, but the Fkh proteins block the pol II from initiating transcription. Thus, the selective antiactivation mechanism impinges on the transcriptional activation program at an earlier step.

### *Conclusions*

We have analyzed divergent regulation by paralogous transcription factors with identical DNA-binding domains. Ace2 is prevented from binding to genes activated solely by Swi5. In contrast, Swi5 binds to, but cannot activate, genes like *CTS1* due to the presence of Fkh1 and Fkh2, which function as selective antiactivators. Antiactivation has been observed in eukaryotes before at the yeast *GAL* genes (Traven *et al*, 2006). Here, the Gal80 protein blocks the activation potential of the Gal4 activator, and antiactivation requires physical interaction between the two proteins. In the presence of galactose, the Gal3 protein overcomes the antiactivation. In contrast, the antiactivation effected by Fkh1/2 onto Swi5 involves two

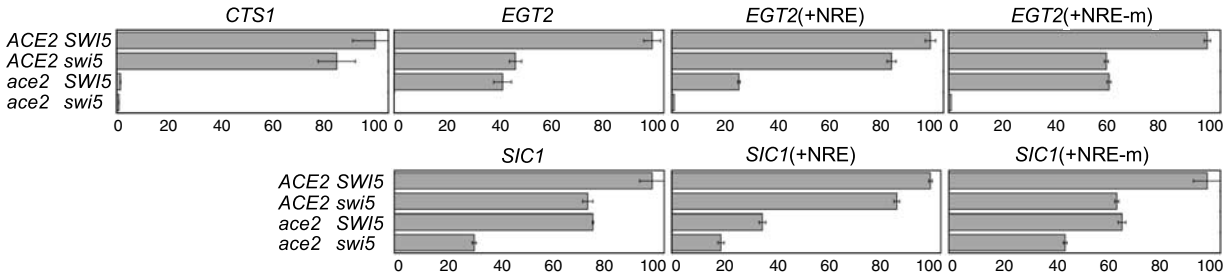



**Figure 8** The NRE element reduces activation by Swi5. RT–PCR assays show *CTS1* is only activated by Ace2, while *EGT2* and *SIC1* is activated by either Ace2 or Swi5. Insertion of the *CTS1* NRE into *EGT2* and *SIC1* (*EGT2*(+NRE) and *SIC1*(+NRE)) reduces *SWI5*-dependent activation, while insertion of a *CTS1* NRE fragment with sequence substitutions through the four four Fkh sites (*EGT2*(+NRE-m) and *SIC1*(+NRE-m)) has no effect on *SWI5*-dependent activation. The RNA values for each set of strains are normalized to the *ACE2 SWI5* strain (defined as 100), and the error bars show the standard deviation of the triplicate PCR reactions.

separate DNA-binding proteins that bind to separate elements on the same promoter and the recruitment of a HDAC. Further work is needed to understand how the Fkh proteins act as selective antiactivators, blocking activation by Swi5 but not by Ace2, and the unique attributes possessed by Ace2 that allow it to overcome this antiactivation.

The Fkh1 and Fkh2 proteins have been previously characterized as activators of the G2/M *CLB2* group of genes, although they may have negative roles at these genes in other cell cycle phases (Hollenhorst *et al*, 2000; Kumar *et al*, 2000; Zhu *et al*, 2000). The *CLB2* group promoters all contain a single Mcm1-binding site in close apposition to a single Fkh consensus site (Althoefer *et al*, 1995b; Pic *et al*, 2000; Boros *et al*, 2003). Here, we show that the Ace2-activated/Fkh-antiactivated group of M/G1 promoters contains multiple Fkh consensus sites, with no discernible Mcm1-binding sequences; additionally, no Mcm1 binding has been detected at these promoters (data not shown, Simon *et al*, 2001). Moreover, Mcm1-Fkh recruit the cell-cycle-regulated transcriptional activator Ndd1 to *CLB2*-group promoters (Koranda *et al*, 2000), but no Ndd1 recruitment to Ace2-regulated promoters has been detected (Simon *et al*, 2001). These differences may explain in part the contrasting earlier function of Fkh proteins at G2/M versus the Ace2 and Swi5-bound genes expressed in late M and G1 phases. We propose that Ace2-activated M/G1 target genes are selectively regulated through a novel antiactivation mechanism, in part comprising HDAC recruitment by the Fkh1/Fkh2 paralogs, determining which target genes are activated by Ace2 as opposed to Swi5 during the ordered cascade of the cell cycle transcriptional program (Simon *et al*, 2001; Pramila *et al*, 2006). It is possible that the acetylation of Swi5 is important for its function as an activator, and the HDACs block this activation; further work will be needed to test this hypothesis.

## Materials and methods

All yeast strains used are listed in Supplementary Table 1 and are isogenic in the W303 background (Thomas and Rothstein, 1989). Standard genetic methods were used for strain construction (Rothstein, 1991; Sherman, 1991), and details on construction of specific alleles are presented in the Supplementary data. Cells were grown at 30°C in YEPD medium (Sherman, 1991). Cell cycle synchronization was performed either by α-factor arrest and release as described (Mitra *et al*, 2006), or by galactose withdrawal and re-addition with a *GAL*::*CDC20* strain (Bhoite *et al*, 2001). A high degree of synchrony was demonstrated by flow cytometry analysis, budding indices, and analysis of cycle-regulated mRNAs (data not shown).

RNA levels were determined with S1 nuclease protection assays as described (Voth *et al*, 2005) or by RT–PCR. For RT–PCR, DNA-free total RNA was purified from mid-log cultures by hot-acid phenol extraction followed by precipitation through CsCl by ultracentrifugation (Ausubel *et al*, 1987). Gene expression levels were quantitated by randomly primed cDNA synthesis with M-MLV Reverse Transcriptase (Invitrogen), followed by real-time quantitative PCR. For microarrays, RNA from *ace2*, *swi5*, and *ace2 swi5* strains were reverse transcribed and each was competitively hybridized with RNA from wild-type strains on Agilent oligonucleotide arrays. Swi5 levels in immunoblots were quantitated using an Odyssey system (Li-Cor).

All ChIPs (except for ChIP–chips) were performed as described (Bhoite *et al*, 2001) using 9E11 (Abcam) or 4A6 (Upstate) monoclonal antibody to the Myc epitope, and 12CA5 antibody to the HA epitope (University of Utah Bioprocessing Resource), anti-histone H3 (07–690, Upstate), anti-histone H3(Ac-Lys14) (07–353, Upstate), anti-acetyl-histone H4 (06–598, Upstate), and antibody coated magnetic beads (Rabbit and Pan Mouse IgG beads, Dynal Biotech). ChIP assays were analyzed as described, either using multiplex PCR (Bhoite *et al*, 2001) or by real-time PCR (Eriksson *et al*, 2004). Oligonucleotides used for ChIP, RT–PCR, or S1 nuclease protection are listed in Supplementary Table 2. Immunoblots of proteins transferred from low-bis-polyacrylamide gel electrophoresis (Whalen and Steward, 1993) were probed with anti-HA, anti-Myc, and anti-PGK 22C5-D8 (Molecular Probes) antibodies, and visualized with an Odyssey Infrared system (LI-COR Biosciences Lincoln, NE). Quantitation of ChIP was calculated as described (Eriksson *et al*, 2004), except that ChIP signals were additionally normalized to the total DNA amount from input chromatin for each sample. Standard deviations for normalized PCR replicates were calculated using Equation (7) of van Kempen and van Vliet (2000).

For the ChIP–chip experiments, eight separate synchrony and immunoprecipitation experiments were performed (four for Ace2 and four for Swi5). A total of 33 ChIP–chip hybridizations were performed, and are listed in Supplementary Table 3. The ChIP–chip experiments were performed as essentially as described (Lieb *et al*, 2001), with complete details provided in the Supplementary data.

***Supplementary data***

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

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