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Opposing roles for Set2 and yFACT in regulating TBP binding at promoters

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Previous work links histone methylation by Set2 with transcriptional elongation. yFACT (Spt16–Pob3 and Nhp6) reorganizes nucleosomes and functions in both transcriptional initiation and elongation. We show that growth defects caused by *spt16* or *pob3* mutations can be suppressed by deleting *SET2*, suggesting that Set2 and yFACT have opposing roles. Set2 methylates K36 of histone H3, and K36 substitutions also suppress yFACT mutations. In contrast, *set1* enhances yFACT mutations. Methylation at H3 K4 by Set1 is required for *set2* to suppress yFACT defects. We did not detect an elongation defect at an 8 kb ORF in yFACT mutants. Instead, *pob3* mutants displayed reduced binding of both pol II and TBP to the *GAL1* promoter. Importantly, both *GAL1* transcription and promoter binding of pol II and TBP are significantly restored in the *pob3 set2* double mutant. Defects caused by an *spt16* mutation are enhanced by either TBP or TFIIA mutants. These synthetic defects are suppressed by *set2*, demonstrating that yFACT and Set2 oppose one another during transcriptional initiation at a step involving DNA binding by TBP and TFIIA.

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Introduction

Eukaryotic DNA is packaged into highly compacted chromatin, limiting the accessibility of transcription factors to DNA. DNA sequences within chromatin can be made accessible to transcription factors in several ways. First, ATP-dependent chromatin remodeling factors can use the energy of ATP to move nucleosomes and expose the DNA sequences for transcription factor binding (Cairns, 2005). Second, variant histones can be inserted into nucleosomes, resulting in chromatin with altered properties (Kamakaka and Biggins, 2005). Third, nucleosomes can be altered by post-translational modifications, including acetylation, methylation and ubiquitylation of lysine residues and phosphorylation of serine residues (Strahl and Allis, 2000; Peterson and Laniel, 2004). These modifications may directly change the properties of chromatin, thereby aiding factor binding, and they may create recognition sites for other factors such as bromodomain- and chromodomain-containing proteins that recognize acetylated and methylated lysines, respectively.

We have been studying a fourth mechanism that enhances accessibility of binding sites, the ATP-independent reorganization of nucleosomes by yFACT (Formosa, 2003). Mammalian FACT contains two subunits, hSpt16 and SSRP1, which are homologous to Spt16 and Pob3 in yeast, except that SSRP1 has a single HMGB DNA-binding motif at its C-terminus that is missing from Pob3 (Wittmeyer and Formosa, 1997; Orphanides *et al*, 1999). The yeast Nhp6 protein, essentially a single HMGB domain, supports the ability of Spt16–Pob3 to function as yFACT both *in vitro* and *in vivo* (Brewster *et al*, 2001; Formosa *et al*, 2001). There is substantial evidence linking yFACT to transcriptional elongation. For example, several yFACT mutants are sensitive to the elongation inhibitor 6-azauracil (6-AU) (Orphanides *et al*, 1998; Formosa *et al*, 2001) and show genetic interactions with known elongation factors (Formosa *et al*, 2002; Squazzo *et al*, 2002). In addition, yFACT physically associates with several elongation factors (Krogan *et al*, 2002; Squazzo *et al*, 2002; Simic *et al*, 2003), human FACT facilitates RNA polymerase II (pol II) elongation through a chromatin template *in vitro* (Orphanides *et al*, 1998), and ChIP and immunolocalization studies show association of FACT with elongating pol II (Mason and Struhl, 2003; Saunders *et al*, 2003).

In addition to a role in transcript elongation, experiments also suggest that yFACT has a role in regulating transcriptional initiation. An *spt16* mutation can change the site of transcriptional initiation (Malone *et al*, 1991), and *Drosophila* FACT associates with the GAGA factor and stimulates chromatin changes at promoters (Shimojima *et al*, 2003). Spt16 inactivation in yeast results in reduced binding of TBP and TFIIB at promoters, *spt16* mutants show strong genetic interactions with mutations affecting TBP and TFIIA, and yFACT facilitates TBP and TFIIA binding to nucleosomal binding sites *in vitro* (Mason and Struhl, 2003; Biswas *et al*, 2005). yFACT can, therefore, enhance the accessibility of DNA sequences in chromatin (Formosa *et al*, 2001), and this is an important component of transcriptional regulation both during initiation and elongation. yFACT also interacts with DNA polymerase α, and with MCM proteins, and it plays an important role in DNA replication (Wittmeyer *et al*, 1999; Gambus *et al*, 2006; VanDemark *et al*, 2006).

Histone proteins are methylated by SET domain-containing proteins, and histone methylation can regulate transcription (Lee *et al*, 2005; Martin and Zhang, 2005). In yeast, histone

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H3 is methylated at K4, K36 and K79 by the Set1, Set2, and Dot1 histone methyl transferases, respectively. It has been suggested that K4 methylation by Set1 facilitates transcriptional elongation, as K4 methylation is enriched in the transcribed regions of actively transcribed genes (Liu *et al*, 2005; Pokholok *et al*, 2005), and Set1 is recruited to elongating RNA polymerase complexes (Ng *et al*, 2003). These observations suggest that Set1 is a positive elongation factor.

Di- and tri-methylation at K36 by Set2 is also found at transcribed open reading frames (Liu *et al*, 2005; Pokholok *et al*, 2005; Rao *et al*, 2005). Set2 also associates with the elongating form of RNA polymerase (Krogan *et al*, 2003; Li *et al*, 2003; Xiao *et al*, 2003). Moreover, *set2* mutants show synthetic growth defects with genes implicated in elongation, consistent with Set2 also being a positive elongation factor (Krogan *et al*, 2003; Li *et al*, 2003). However, several observations are more consistent with a negative role for Set2 in regulating transcription initiation. K36 methylation by Set2 is required to recruit the Rpd3S histone deacetylase complex through its Eaf3 chromodomain subunit, and deacetylation by Rpd3S may be required to restore chromatin to the repressed pretranscribed state (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). Set2 represses transcription when tethered to a heterologous promoter, indicating a direct negative effect on initiation (Strahl *et al*, 2002). Additionally, expression of a mutant *GAL4* promoter lacking its UAS element is very low, but can be increased either by a *set2* mutation or a histone H3 K36R substitution, suggesting that modification of H3 by Set2 inhibits initiation (Landry *et al*, 2003). Importantly, although several studies have shown greater K36 methylation at open reading frames (Pokholok *et al*, 2005; Rao *et al*, 2005), it is clear that K36 methylation also occurs in promoter regions (Xiao *et al*, 2003). Set2 therefore has complex and perhaps opposite effects on different stages of transcriptional regulation.

In this report, we show that yFACT and K36 methylation by Set2 have opposing roles in regulating transcription. Surprisingly, we find no evidence that mutations affecting yFACT and Set2 influence transcriptional elongation rates. Instead, our results show that yFACT and histone methylation by Set2 regulate, in opposite ways, binding of both pol II and TBP to promoters.

Results

Set1 methylation at histone H3 K4 supports the function of yFACT

SPT16 and *POB3* are essential genes, and mutant alleles with distinct phenotypes have been isolated (Malone *et al*, 1991; Rowley *et al*, 1991; Schlesinger and Formosa, 2000; Formosa *et al*, 2001). We chose the *spt16-11* and *pob3(L78R)* alleles for these studies because they display the Spt-phenotype from inappropriate TATA element usage, and they are sensitive to elevated temperatures, to the dNTP synthesis inhibitor hydroxyurea (HU), and to the transcription elongation inhibitor 6-AU. Thus, the phenotypes of the *spt16-11* and *pob3(L78R)* alleles suggest that they have defects in transcriptional initiation, transcriptional elongation, as well as in replication of DNA.

We previously showed that some yFACT mutations are synthetically lethal with some mutations in histone H3 and H4, including deletions of the N-terminal tails and mutations of certain acetylatable lysine residues (Formosa *et al*, 2002; VanDemark *et al*, 2006). Here, we look for genetic interactions between yFACT mutations and H3 mutations in methylated lysines K4 and K79, and acetylated site K23. Strains with deletions of both sets of chromosomal genes encoding histone H3 and H4, and carrying a YCp-*URA3* plasmid with the wild-type *HHT2–HHF2* genes were constructed. Plasmids with either wild-type or mutant *HHT2-HHF2* alleles were introduced into these strains by transformation, and the ability of transformants to grow on media with FOA was assessed. *URA3+* strains cannot grow on FOA, and thus growth demonstrates that the wild-type histone genes on the YCp-*URA3* plasmid can be lost with the introduced plasmid supporting viability.

As shown in Figure 1A, introducing plasmids with wild-type histones, H3(K4R), H3(K23R), or H3(K79R) into a wild-type strain results in healthy growth, while the empty vector does not. We conclude that these H3 mutations support viability in a wild-type strain, the H3(K23R) mutation shows a modest growth defect in combination with either an *spt16-11* (Figure 1B) or a *pob3(L78R)* (Figure 1C) mutation, and H3(K79R) does not affect growth of these mutants. The

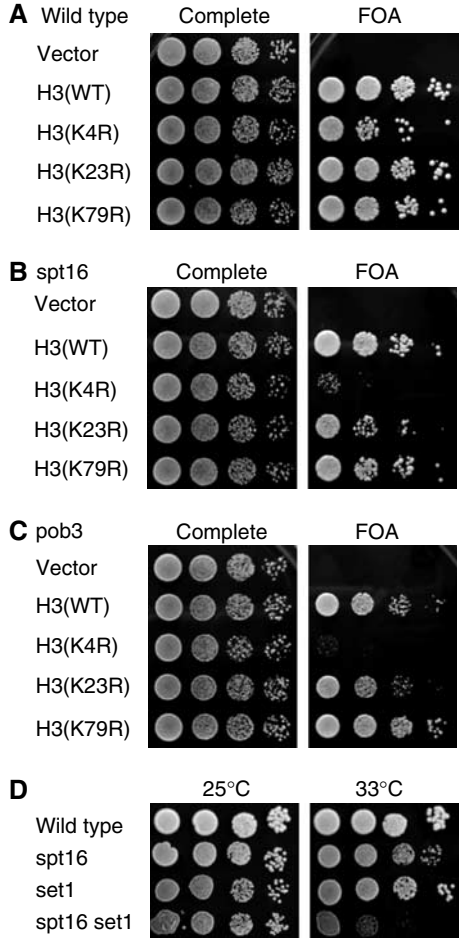


Figure 1 Histone H3(K4R) substitutions enhance the defects caused by *spt16* and *pob3* mutations. (**A**) Strain DY7803 was transformed with a YCp-TRP1 plasmid with wild-type histone H4 gene and the indicated histone H3 mutation, and dilutions were plated on the indicated medium for 2 days at 33°C. (**B**) As in panel (A), except the strain is DY7809. (**C**) As in (A) except the strain is DY7818 and dilutions were incubated for 3 days at 25°C. (**D**) Dilutions of strains DY150, DY8788, DY8875, and DY9206 were plated on complete medium at 25°C for 3 days or at 33°C for 2 days.

H3(K4R) mutation has a more striking effect, showing a strong synthetic defect when combined with either *spt16* or *pob3*. Lysine 4 of histone H3 is methylated by the Set1 enzyme (Briggs *et al*, 2001), and thus we predict a similar effect from a *set1* mutation. We constructed an *spt16 set1* double mutant and found it to be viable at 25°C, but lethal at 33°C (Figure 1D). We were unable to construct a *pob3 set1* double mutant, as it was lethal at all temperatures tested. We conclude that the function of yFACT is strongly dependent on methylation of histone H3 at K4 by Set1.

Absence of Set2 methylation at histone H3 K36 suppresses temperature sensitivity caused by yFACT mutations

In contrast with our results with the K4R mutation, we found that mutations at histone H3 K36 suppress growth defects associated with yFACT mutations. The *spt16-11* mutant does not grow at 35°C, as evidenced by its failure to grow on FOA when containing a plasmid with wild-type histone genes (Figure 2A). However, the *spt16* mutant grows on FOA if the plasmid contains either a K36R or a K36A mutation in histone H3. Similarly, a strain with the *pob3(L78R)* allele is unable to grow at 30°C, but the H3 K36R or K36A mutations suppress this growth defect (Figure 2B). To verify that this apparent growth suppression was not an artefact of growth on FOA-containing medium, we used a plasmid shuffle at 25°C to evict the YCp-*URA3* plasmid, obtaining strains with a YCp-*TRP1* plasmid with either wild-type histone genes or a derivative with the H3(K36A) substitution. The *spt16* mutant with wild-type histone H3 is unable to grow at 35°C, but the K36A substitution suppresses (Figure 2C). Similarly, histone H3(K36A) suppresses the *pob3* mutant (Figure 2D).

The Set2 enzyme methylates K36 of histone H3 (Strahl *et al*, 2002), so if methylation at this site by Set2 is the cause of the suppression, a *set2* mutation should have a similar phenotype to an H3 K36 mutation. As shown in Figure 2E and F, a *set2* gene deletion also suppresses the temperature sensitivity of the *spt16* and *pob3* strains. These observations clearly indicate that Set2 methylation of histone H3 at K36 has an opposing role to that of yFACT in supporting viability.

Opposing roles of Set1 and Set2 methyltransferases

The temperature-sensitive growth defect in an *spt16* mutant is affected by *set1* and *set2* mutations, but in opposite directions. To examine the epistasis relationships, we constructed an *spt16 set1 set2* triple mutant. The results in Figure 3A show that the triple mutant has a marked growth defect, although not quite as severe as the *spt16* mutant or the *spt16 set1* double mutant. We next constructed a plasmid with both histone H3 K4R and K36R mutations and tested it in the *spt16* and *pob3* mutants. The results in Figure 3B show that the H3(K4R,K36R) double mutant is synthetically lethal with both *spt16* and *pob3*, the same phenotype seen with K4R. Thus, the K4R mutation is epistatic to K36R. We conclude that the absence of Set2 methylation at H3 K36 can only suppress the yFACT defects when methylation by Set1 occurs at H3 K4.

Histone mutations affect growth in nhp6ab mutant strains

In addition to Spt16 and Pob3, yFACT contains Nhp6, a small HMG protein required for nucleosomal binding by Spt16–Pob3 (Formosa *et al*, 2001). Nhp6 is encoded by two redundant genes, *NHP6A* and *NHP6B*, and the *nhp6ab* double mutant shows growth defects. We next examined the effect of histone mutations on an *nhp6ab* strain. Similar to the results with *spt16* and *pob3* mutants, the H3(K4R) mutation

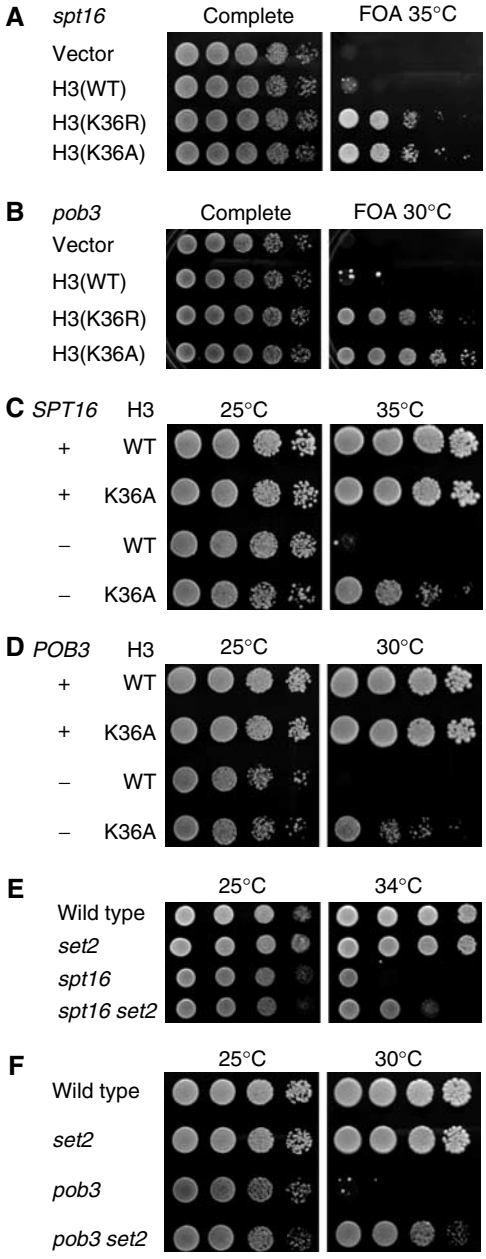


Figure 2 Histone H3(K36) substitutions and *set2* mutations suppress *spt16* and *pob3* mutations. (**A**) Strain DY7809 was transformed with a YCp-TRP1 plasmid with wild-type histone H4 gene and the indicated histone H3 mutant, and dilutions were plated on complete medium (2 days) or FOA medium (3 days) at 35°C. (**B**) As in (A), except the strain is DY7818 and dilutions were plated on complete medium (3 days) or FOA medium (5 days) at 30°C. (**C**) Dilutions of Strains DY8862, DY8864, DY8865, and DY8867 were plated on complete medium at the indicated temperature for 3 days. (**D**) Dilutions of strains DY8862, DY8864, DY10468, and DY10469 were plated on complete medium at the indicated temperature for 3 days. (**E**) Dilutions of strains DY150, DY8690, DY8787, and DY8790 were plated on complete medium at the indicated temperature for 2 days. (**F**) Dilutions of strains DY150, DY8690, DY8881, and DY8878 were plated on complete medium at 25°C for 2 days or at 30°C for 3 days.

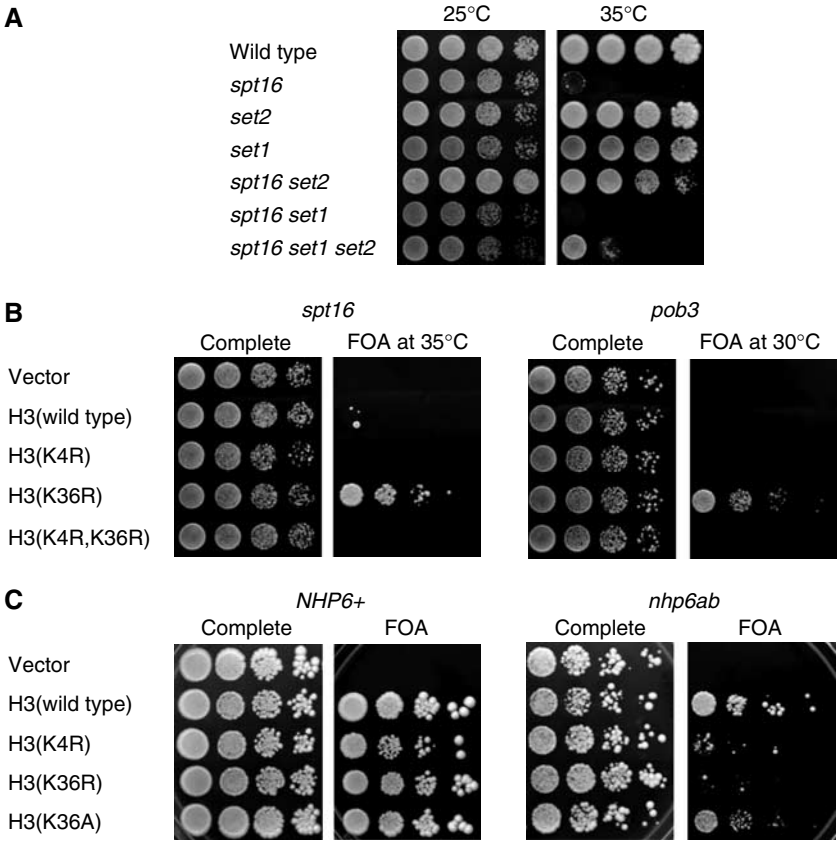


Figure 3 *set1* is epistatic to *set2* in genetic interactions with *spt16*. (**A**) Dilutions of strains DY150, DY8787, DY8690, DY8875, DY8777, DY9178, and DY9180 were plated on complete medium at 25°C for 2 days or at 35°C for 3 days. (**B**) Strains DY7809 and DY7818 were transformed with a YCp-TRP1 plasmid with wild-type histone H4 gene and the indicated histone H3 mutant, and dilutions were plated and incubated as follows: *spt16* on complete, 2 days at 25°C, *spt16* on FOA, 4 days at 35°C, *pob3* on complete, 3 days at 25°C, and *pob3* on FOA, 5 days at 30°C. (**C**) As in (B), except the strains are DY7803 and DY7142, and dilutions were plated on the indicated medium at 33°C for 4 days.

caused a severe synthetic defect with *nhp6ab* (Figure 3C) and H3(K79) mutations had no effect (not shown). However, the H3(K36) substitutions had a markedly different effect in the *nhp6ab* mutant; instead of the suppression seen with *spt16* and *pob3*, the K36 mutations inhibited growth of the *nhp6ab* strain. A synthetic growth defect is also seen when a *set2* mutation is introduced into a *nhp6ab* strain (data not shown). In addition to its role in yFACT, Nhp6 has been shown to interact with other chromatin proteins, including Swi/Snf, RSC, and Ssn6/Tup1 (Szerlong *et al*, 2003; Biswas *et al*, 2004; Fragiadakis *et al*, 2004) and to play a role in transcription by RNA polymerase III (Kassavetis and Steiner, 2006). These additional roles for Nhp6 could explain why the H3(K36) mutations have such markedly different effects in the *nhp6ab* strain compared to the *spt16* and *pob3* mutants.

A variety of yFACT mutant defects are suppressed by set2

spt16-11 mutants are defective for growth on media containing 6-AU (Formosa *et al*, 2001). 6-AU is a uracil analog that causes imbalances in the pools of rNTPs (Exinger and Lacroute, 1992; Shaw and Reines, 2000), and many strains with defects in transcriptional elongation are sensitive to 6-AU. We determined whether the 6-AU sensitivity caused by *spt16* could be suppressed either by a histone mutation or by a *set2* mutation. As shown in Figure 4A, the 6-AU sensitivity of the *spt16* strain (line 4) is suppressed by a K36A substitution in histone H3 (line 6). A *set2* mutation similarly suppresses the 6-AU sensitivity caused by *spt16* (Figure 4B). We note that *set2* mutants display slightly higher 6-AU resistance than wild-type strains, consistent with previous reports (Adhvaryu *et al*, 2005; Keogh *et al*, 2005; Kizer *et al*, 2005). Thus, *spt16* and *set2* mutants have opposite responses to 6-AU, suggesting that yFACT and Set2 have opposing roles in transcriptional elongation. However, sensitivity to 6-AU does not necessarily demonstrate a role in transcriptional elongation; a mutation in the *SNR6* promoter that reduces expression of the U6 small nuclear RNA causes 6-AU sensitivity (Eriksson *et al*, 2004).

Based on the observation that a *set2* mutation suppresses the temperature- and 6-AU-sensitive phenotypes associated with yFACT mutations, we tested whether *set2* can also suppress other synthetic defects observed with yFACT mutants. An *spt16* mutation displays synthetic defects with mutations in either the *GCN5* or the *ELP3* histone acetyltransferase genes, or with the *nhp6ab* double mutant (Formosa *et al*, 2002), and a *set2* mutation suppresses all of these synthetic growth defects (Supplementary Figure S1). It has been proposed that the Isw1 chromatin remodeling factor has a role in transcriptional elongation (Morillon *et al*, 2003). A *spt16 isw1* double mutant shows a synthetic growth defect at 33°C, and this is suppressed by *set2* (Figure 4C). These suppression results support the idea that yFACT and Set2 have opposing roles in multiple functions.

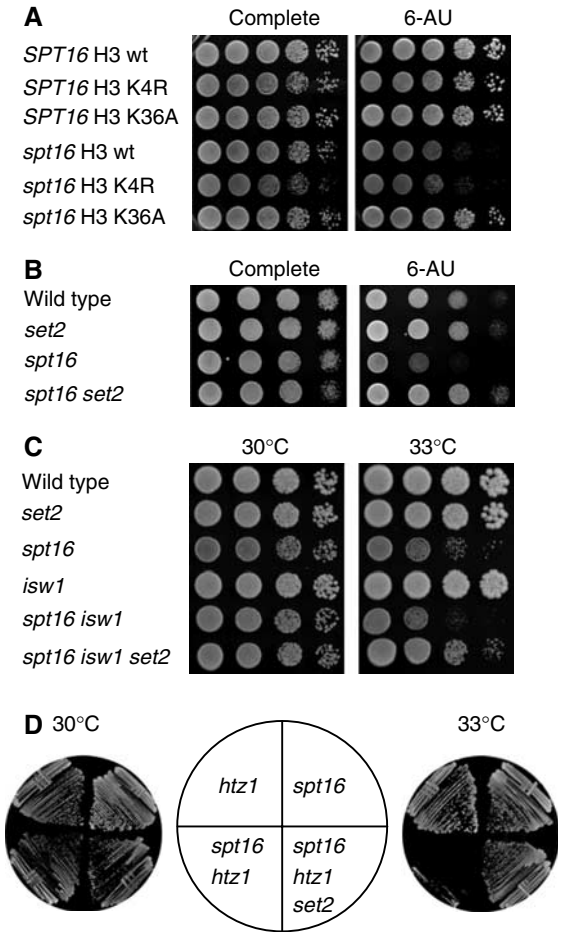


Figure 4 *set2* suppress *spt16* phenotypes. (**A**) *set2* suppresses the 6-AU sensitivity caused by an *spt16* mutation. Dilutions of strains DY8883, DY8884, DY8885, DY8886, DY8887, and DY8888 were plated at 25°C on complete medium for 2 days or on medium lacking uracil containing 50 μg/ml 6-azauracil (6-AU) for 4 days. (**B**) H3(K36A) suppresses the 6-AU sensitivity caused by an *spt16* mutation. As in (A), except the strains are DY3398, DY8789, DY8788, and DY8790. (**C**) *set2* suppresses the *spt16 isw1* synthetic growth defect. Dilutions of strains DY150 (wild type), DY8107 (*spt16*), DY8690 (*set2*), DY8235 (*isw1*), DY9022 (*spt16 isw1*), and DY9029 (*spt16 isw1 set2*) were plated on complete medium at the indicted temperature for 3 days. (**D**) *set2* suppresses the *spt16 htz1* synthetic growth defect. Strains DY7836, DY9808, DY9805, and DY8107 were plated on complete medium at the indicated temperature for 2 days.

The H2A.Z histone variant of H2A in yeast, encoded by *HTZ1* gene, has diverse functions (Dryhurst *et al*, 2004). We constructed an *spt16 htz1* double mutant strain, and observed synthetic lethality at 33°C (Figure 4D). This growth defect is also suppressed by a *set2* mutation. Importantly, genome wide studies show that the Htz1 protein localizes preferentially at the promoter regions of genes (Li *et al*, 2005; Raisner *et al*, 2005; Zhang *et al*, 2005). This promoter localization of Htz1, and the genetic interactions seen here, suggest that Set2 and yFACT might have opposing roles at promoter regions, in addition to their proposed elongation functions.

Specificity of set2 suppression

We also tested whether *set1* or *set2* mutations also affect other factors thought to be involved in transcriptional elongation. We constructed double mutant strains, combining either a *set1* or a *set2* mutation with disruptions in *PAF1*, *CDC73*, *DST1*, *SPT4*, or *ELP3*. The double mutants with *set1* or *set2* were examined for growth phenotypes, including sensitivity to temperature and 6-AU. There are some instances of suppression and some of synthetic defects (Supplementary Figure S2A). However, these elongation mutants do not all show suppression with *set2* and synthetic defects with *set1*, and thus the effect appears to be specific to *spt16* and *pob3*.

spt16 mutants also cause an Spt-phenotype, altering transcription start sites from the *his4-912δ* and *lys2-128δ* alleles and conferring a His+ Lys+ phenotype (Malone *et al*, 1991). Interestingly, *spt16 set2* strains are still Spt-, and thus *set2* does not suppress this phenotype (data not shown). Similarly, the synthetic growth defect seen in a *spt16 rpd3* double mutant (Formosa *et al*, 2002) is not suppressed by *set2* (data not shown). However, we do see suppression by *set2* of the Spt-phenotype seen in a *gcn5* mutant with the *lys2-173R2* allele (Supplementary Figure S1D). A *set2* deletion, therefore, does not suppress all elongation defects, and it also does not suppress all of the defects caused by yFACT mutations.

Pob3 and Set2 regulate GAL1 induction in opposing ways

Mason and Struhl (2003) used a *GAL1-YLR454w* reporter, with the *GAL1* promoter inserted upstream of the nonessential 8 kb *YLR454w* gene, to show that yFACT associates with open reading frames during transcription. We constructed *pob3* and *set2* strains with this *GAL1-YLR454w* allele and measured *YLR454w* mRNA levels by S1 nuclease protection assays following induction of the *GAL1* promoter. The results in Figure 5A show a rapid increase in *YLR454w* mRNA in the wild-type strain, as expected. The *pob3* mutant strain is markedly defective in inducing *YLR454w* mRNA from the *GAL1* promoter (Figure 5A), and a similar defect is seen in induction of the native *GAL1* gene in the *pob3* mutant (Figure 5B). Importantly, deletion of *SET2* ameliorates the transcriptional defect significantly at *GAL1-YLR454w* (Figure 5A), and completely at *GAL1* (Figure 5B) (compare *pob3* and *pob3 set2*). Additionally, we examined other genes and found either an *spt16* or *pob3* mutation could reduce expression, and a *set2* mutation partially suppresses these defects (Supplementary Figure S3A). These observations imply that Set2 and yFACT have opposing roles in regulating transcription.

While a *set2* mutant caused decreased induction of *GAL1-YLR454w*, it displayed increased expression before induction (Figure 5C). Set2 is, therefore, needed both for repression of the *GAL1* promoter in this context and for normal induction of expression, underscoring the complexity of the role of Set2 in transcription. Notably, Set2 is not needed for full induction of the native *GAL1* message (Figure 5B). It is possible that the large size of the 8 kb *YLR454w* transcription unit places a larger demand on Set2 function than the 1.6 kb *GAL1* gene.

Defect in pol II binding to the GAL1 promoter in pob3 mutants

We used the *GAL1-YLR454w* reporter to assess the rate of pol II elongation in wild-type and mutant strains in several ways. In the first assay, we took RNA samples every 10 min following galactose induction, and used probes for S1 nuclease

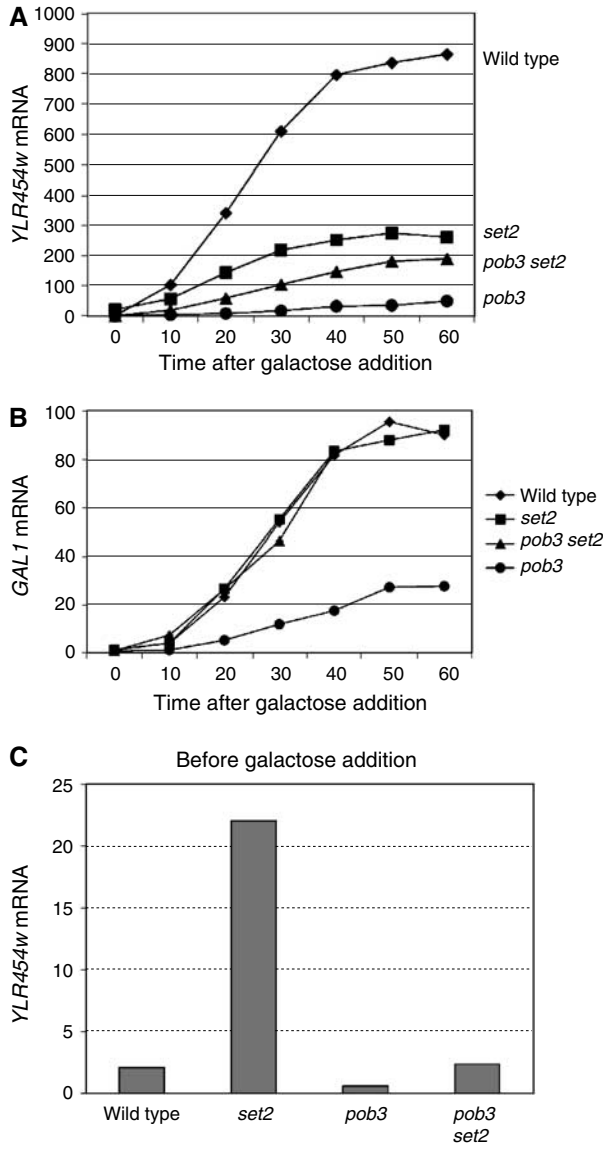


Figure 5 A *set2* mutation reverses the poor induction of *GAL1* caused by a *pob3* mutation. Strains DY9591, DY9976, DY9972, and DY9974 were grown on YP medium with 2% raffinose. Galactose was added to 2% concentration, and samples were taken at 10 min intervals and mRNA measured by S1 nuclease protection. (**A**) *YLR454w* mRNA levels from the *GAL1-YLR454w* allele. (**B**) *GAL1* mRNA levels. (**C**) *YLR454w* mRNA levels before galactose induction.

protection assays specific to the 5′ and 3′ ends of the gene. The time between appearances of mRNA sequences corresponding to the 5′ and 3′ ends gives an indication of how long it takes for pol II to traverse the 8 kb gene. We do not see a significant difference in this time in the wild-type, *set2*, *pob3*, and *pob3 set2* strains (Supplementary Figure S4). In a second assay to measure elongation rates, glucose was added to cells growing in galactose to shut down expression of *GAL1-YLR454w,* and chromatin immunoprecipitation (ChIP) was used to measure Pol II occupancy during the last wave of transcription down the gene (Mason and Struhl, 2005). This assay did not show any elongation defect in *pob3* mutants (Supplementary Figure S5). Thus, the marked defect in *GAL1-YLR454w* expression in *pob3* mutants is not due to an elongation defect. Of course, *pob3* mutants may be defective for elongation at other genes.

We also used ChIP assays to measure pol II levels along the *YLR454w* gene following galactose induction. Figure 6A shows a map of the *GAL1-YLR454w* reporter, and four regions amplified with specific primers corresponding to the *GAL1-YLR454w* promoter, 1 kb downstream of start codon, the middle of the *YLR454w* ORF (+3600), and the 3′ end of the gene (+7800). At time intervals following galactose induction, samples were harvested, treated with formaldehyde to crosslink, and processed for ChIP. The results of the pol II ChIP from the wild-type strain are shown in Figure 6B. As expected, pol II occupancy increases throughout the gene during induction. Pol II binding to the promoter is not saturated during this time course, unlike previous results (Bryant and Ptashne, 2003); there are important differences in media and cell concentration between the two studies. A delay is seen in pol II occupancy at +1000 and downstream, reflecting the amount of time required to initiate and progress through the ORF. For example, occupancy reaches a 30-fold ratio after about 8 min at the promoter, 14 min at +1000, and 22 min at +7800. Notably, the time course is similar for a *pob3* mutant, although the overall occupancy is much lower (Figure 6C). This is consistent with similar elongation rates at *GAL1-YLR454w* in wild-type and *pob3* strains; similar results are seen with *set2* and *pob3 set2* strains (data not shown).

Examination of the wild-type strain shows that occupancy at the promoter is much higher than it is at any point within the ORF at all time points. Plotting the Pol II occupancy at the 50 min time point clearly shows this difference (Figure 6D). Greater occupancy at the promoter suggests that recruitment is faster than the transition from initiation to elongation under these strongly inducing conditions. Pol II occupancy at all sites decreases in both *set2* and *pob3* mutants, so Set2 and yFACT are both involved in recruiting Pol II to this promoter. Interestingly, among the mutants, only the *pob3 set2* strain has slightly higher pol II occupancy at the promoter than at the ORF. Loss of the higher accumulation of Pol II at the promoter compared to the ORF in the mutants might indicate that Set2 and yFACT cooperate to impose a barrier or selective elongation switch between recruitment and initiation or elongation by Pol II. This functional cooperation between Set2 and yFACT contrasts with the opposing roles detected above.

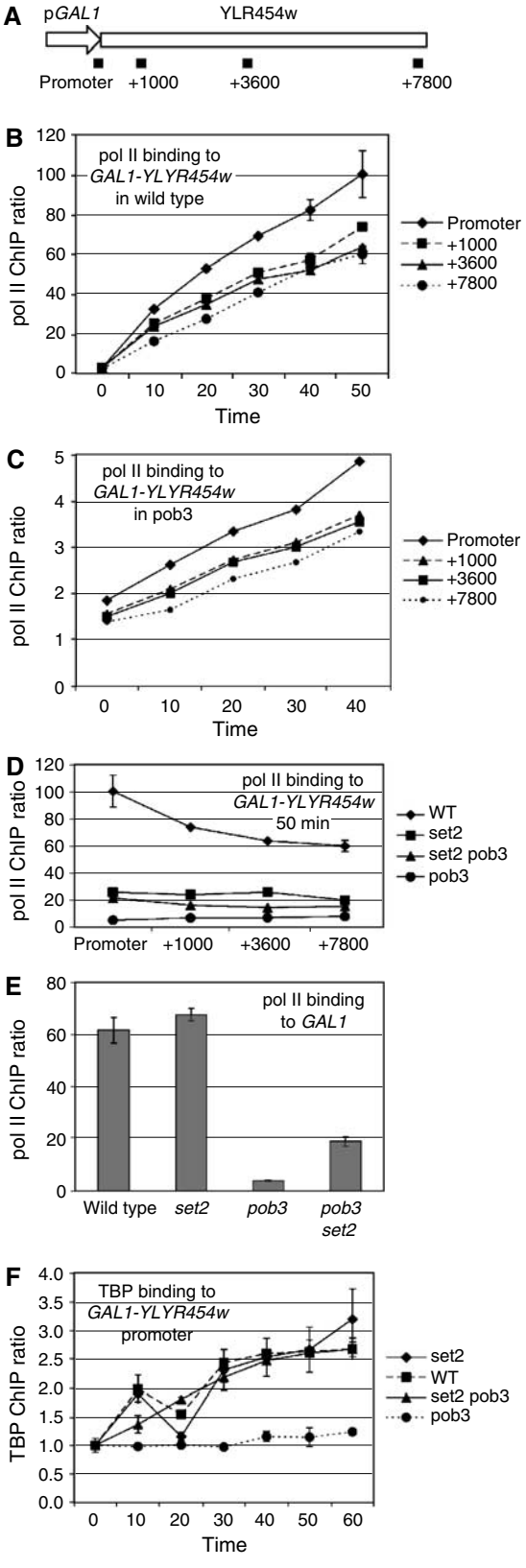
Importantly, while a *pob3* strain has a severe defect in pol II occupancy at the at *GAL1-YLR454w* promoter, the *set2 pob3* double mutant has a less severe defect, similar to the *set2* single mutant (Figure 6D). The same effect is seen at the native *GAL1* promoter, where the *set2* mutation does not by itself cause diminished pol II occupancy (Figure 6E). Deletion of *SET2,* therefore, at least partially restores the ability of a *pob3* mutant to recruit pol II to a promoter.

Defective TBP binding in pob3 mutants is suppressed by set2

Pol II is typically recruited to promoters by the TATA-binding protein TBP. We have previously shown that yFACT can play a role in transcriptional initiation through regulation of TBP binding (Biswas *et al*, 2005), and we therefore examined TBP binding to the *GAL1-YLR454w* promoter in these mutants. As shown in Figure 6F, TBP binding to *GAL1-YLR454w* is essentially eliminated in the *pob3* mutant but is largely restored in

the *pob3 set2* double mutant strain. We also found TBP binding at other promoters is reduced in a *pob3* mutant, and increased TBP binding is seen in the *pob3 set2* strain compared to the pob3 single mutant (Supplementary Figure S3B). Thus, TBP binding to the *GAL1* promoter is stimulated by yFACT, and the *pob3* defect can be suppressed by a *set2* mutation.

Pol II binding at *GAL1-YLR454w* is markedly reduced in the *set2* and *pob3 set2* strains, while TBP binding is at wild-type levels. Thus TBP binding is not sufficient to direct pol II recruitment, and other factors such as TFIIA or TFIIB may be involved.



The synthetic lethality between spt16 and either TBP or TFIIA mutations is suppressed by set2

We previously demonstrated genetic interactions between *spt16* and TBP mutations (Biswas *et al*, 2005). We showed that combining mutant Spt16 and mutant TBP proteins in the same cell results in lethality, using a plasmid shuffle assay to introduce mutant alleles into a strain lacking both *SPT15* (which encodes TBP) and *SPT16*.

We have repeated this experiment, now including an *spt15*Δ *spt16*Δ *set2* YCp-*URA3*-TBP-Spt16 strain. As shown in Figure 7A, certain combinations of Spt16 and TBP result in lethality in *SET2* strains, but a *set2* mutation allows these combinations of Spt16 and TBP to be viable. For example, cells with *spt16-11* and TBP(E93G) cannot lose the YCp-*URA3*-TBP-Spt16 plasmid with the wild-type genes, as evidenced by the failure to grow on FOA, demonstrating the synthetic lethality. In contrast, the *set2* mutant with *spt16-11* and TBP(E93G) can grow on FOA. Thus, *set2* suppresses the synthetic lethality between *spt16* and TBP.

We also showed synthetic lethality between *spt16* and TFIIA mutations (Biswas *et al*, 2005). Yeast TFIIA protein is composed of two subunits, Toa1 and Toa2. Based on the TBP–TFIIA crystal structure, Ozer *et al* (1998) generated *toa2* mutations at the TBP–TFIIA interface. Although these mutations in *TOA2* eliminate TBP–TFIIA interactions with *in vitro* binding assays, the *toa2* mutants are viable, presumably because other factors present in cells facilitate TBP–TFIIA interaction and DNA binding. For example, Toa2(W76) is required for cooperative DNA binding with TBP *in vitro*, but a *toa2*(W76A) mutant is viable in an otherwise wild-type strain (Ozer *et al*, 1998; Biswas *et al*, 2005). The *toa2*(W76A) allele is lethal in an *spt16* mutant (Figure 7B). However, *toa2*(W76A) is viable in the *spt16 set2* strain, and thus *set2* suppresses the synthetic lethality between *spt16* and TFIIA mutations.

Figure 6 A *pob3* mutation reduces pol II and TBP binding, and binding is restored in a *pob3 set2* strain. Strains DY9591, DY9976, DY9972, and DY9974 were grown on YP medium with 2% raffinose. Galactose was added to 2%, and samples were taken at 10 min intervals and processed for ChIP analysis to measure pol II and TBP binding. (**A**) Map of the *GAL1-YLR454w* allele showing the positions of the PCR primers at the promoter and within the gene. (**B**) Kinetics of pol II binding following galactose induction at different *GAL1-YLR454w* regions in a wild-type strain. Error bars show variance among replicate PCRs. (**C**) Kinetics of pol II binding following galactose induction at different *GAL1-YLR454w* regions in a *pob3* strain. (**D**) Distribution of pol II at 50 min following galactose induction at different *GAL1-YLR454w* regions in four different strains. Error bars show variance among replicate PCRs. (**E**) Pol II binding to the native *GAL1* promoter at 30 min following galactose induction in four different strains. Error bars show variance among replicate PCRs. (**F**) TBP binding to the *GAL1-YLR454w* promoter following galactose induction in four different strains. ChIP values were normalized to binding at $t = 0$. Error bars show variance among replicate PCRs.

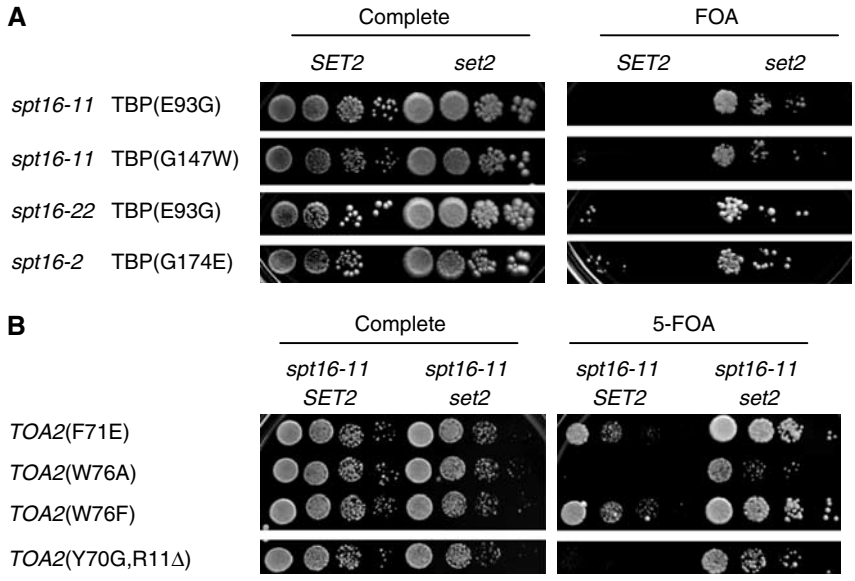


Figure 7 A *set2* mutation suppresses the synthetic lethality of an *spt16* mutation with either TBP or TFIIA mutations. (**A**) Strains DY8552 (indicated as '*SET2*') and DY10065 (indicated as '*set2*') were transformed with YCp-*TRP1* plasmid encoding a TBP mutant, and dilutions were plated on complete or FOA medium at 33°C for 3 days. (**B**) Strains DY8700 (indicated as '*SET2*') and DY10212 (indicated as '*set2*') were transformed with a YCp-*LEU2* plasmid with the indicated *toa2* mutant, and dilutions were plated on complete medium at 25°C for 2 days and on FOA medium at 30°C for 4 days, except the *TOA2*(Y10G,R11Δ) strains were incubated on FOA medium at 33°C.

Suppression of the synthetic lethality between *spt16* and TBP or TFIIA mutants by deletion of *SET2* strongly supports our hypothesis that yFACT and Set2 have opposing roles in supporting binding of TBP and TFIIA to promoters.

Discussion

Spt16 and Pob3, along with Nhp6, comprise the yFACT complex that can reorganize chromatin structure. *SPT16* and *POB3* are essential genes, and *spt16* and *pob3* point mutants have been isolated with phenotypes including temperature-sensitive growth, sensitivity to 6-AU, and synthetic lethality with certain transcription factor mutations. We find that a *set2* deletion, eliminating the enzyme that methylates lysine 36 on histone H3, suppresses all of these phenotypes. Additionally, a histone H3 mutation, replacing the lysine at position 36 with either an alanine or an arginine residue, also suppresses *spt16* and *pob3* mutations. We conclude that methylation on histone H3 at K36 by Set2 acts in opposition to the chromatin changes facilitated by yFACT. *spt16* and *pob3* mutants also cause initiation at aberrant sites, leading to the Spt- phenotype. This defect was not suppressed by *set2*, indicating that not all functions of yFACT are opposed by Set2.

Both Set2 and yFACT have been implicated in regulating transcriptional elongation. Both localize preferentially to transcribed regions compared to promoters (Krogan *et al*, 2003; Mason and Struhl, 2003; Saunders *et al*, 2003), Set2 associates with hyperphosphorylated pol II (Li *et al*, 2003; Xiao *et al*, 2003), and several studies have shown greater K36 methylation at open reading frames compared to promoters (Xiao *et al*, 2003; Pokholok *et al*, 2005; Rao *et al*, 2005). Additionally, both *spt16* and *set2* mutations show genetic interactions with known elongation factors (Formosa *et al*, 2002; Squazzo *et al*, 2002; Krogan *et al*, 2003; Li *et al*, 2003). We looked for defects in transcriptional elongation in a *pob3* mutant, using a strain with the *GAL1* promoter inserted upstream of the nonessential 8 kb *YLR454w* gene. We found no evidence for a transcriptional elongation defect at *GAL1-YLR454w* in *pob3* mutants, but *pob3* mutants may affect elongation at other genes. We did find reduced expression of both *GAL1* and *GAL1-YLR454w* in the *pob3* mutant. Importantly, expression was restored in the *pob3 set2* double mutant.

ChIP experiments indicate that yFACT and Set2 act at the *GAL1* promoter at the level of transcriptional initiation. Following galactose induction, there is reduced binding of both pol II and TBP at the *GAL1* promoter in the *pob3* mutant; binding of both factors is increased in the *pob3 set2* strain. These experiments suggest that yFACT facilitates TBP binding, at least at some promoters, and Set2 opposes this effect. How does Set2 inhibit TBP binding at *GAL1*? One possibility is that histone methylation by Set2 inhibits binding of a transcriptional coactivator complex that stimulates TBP binding. The SAGA complex is recruited to *GAL1* and promotes TBP binding (Dudley *et al*, 1999). We have analyzed SAGA binding at the *GAL1 UAS* by ChIP, but we did not observe any significant difference in SAGA binding in strains differing at the *POB3* and *SET2* loci (data not shown). Chromodomains bind to methylated histone residues, and it is possible that methylated K36 residues at the *GAL1* promoter encourage binding of a chromodomain-containing transcription factor that regulates TBP binding.

Our results strongly support a role for K36 methylation by Set2 in decreasing TBP binding to promoters. There are two earlier studies that support a repressive role for Set2 at promoters. A Set2–LexA fusion protein strongly represses transcription when tethered to a promoter with a lexA-binding site (Strahl *et al*, 2002). This repression by the Set2–LexA fusion protein was reduced by point mutations in the Set2

catalytic domain. In a separate study, the weak expression from a *GAL4* promoter lacking its UAS element could be suppressed by either a *set2* mutation or a K36R substitution in histone H3 (Landry *et al*, 2003). This suggests that Set2 can repress transcription from promoters.

Set2 and yFACT are believed to function in transcriptional elongation, although we did not see an effect of mutations on the elongation rate at one specific gene. Our results strongly suggest that both Set2 and yFACT function at initiation of transcription by regulating DNA binding by TBP, at least at the *GAL1* promoter. We previously demonstrated that *spt16* mutants are synthetically lethal with point mutations in either TBP or TFIIA (Biswas *et al*, 2005). Importantly, a *set2* mutation can suppress this synthetic lethal interaction. Additionally, *in vitro* studies show that yFACT can facilitate binding of TBP and TFIIA to a nucleosomal TATA site that is normally refractory to binding (Biswas *et al*, 2005). Biochemical studies have shown that the TFIIS factor (encoded by *DST1* in yeast) facilitates elongation by pol II. However, it was recently shown that TFIIS binds to the *GAL1* promoter (Prather *et al*, 2005). Additionally, a *dst1* mutation affected the kinetics of *GAL1* induction, and reduced the association of both TBP and pol II to the *GAL1* promoter (Prather *et al*, 2005), and a further decrease in promoter occupancy by basal factors is seen when a *dst1* mutant is treated with 6-AU (Mason and Struhl, 2005). It has been suggested that a decrease in elongation slows promoter clearance, leading to destabilization of the preinitiation complex (Mason and Struhl, 2005). It is intriguing that TFIIS, Set2 and yFACT, all proposed as elongation factors, regulate binding of TBP at *GAL1*.

Although a *set2* mutation suppresses defects caused by yFACT mutations, we find that combining a *set1* mutation with either *spt16* or *pob3* results in synthetic defects. Set1 and Set2 methylate histone H3 at different residues, K4 and K36, respectively. A histone H3 K4 substitution enhances yFACT defects, while a substitution at K36 suppresses these defects. We examined the epistasis relationships by constructing an *spt16 set1 set2* triple mutant, and by testing a histone H3(K4R, K36R) double mutant in *spt16* and *pob3* strains. The results show that the absence of K36 methylation is not sufficient to suppress the yFACT mutants. It is the combination of methylated K4 and unmethylated K36 at histone H3 that suppresses the yFACT defects.

How do the presence or absence of methylated lysine residues on histone H3 produce such marked effects on growth of cells with a partially defective yFACT complex? Chromodomain-containing proteins can bind to methylated lysine residues. One group has reported that yeast Chd1 binds to methylated K4 (Pray-Grant *et al*, 2005), while another group finds that human, but not yeast Chd1, is capable of binding to methylated K4 (Sims *et al*, 2005). It is possible that in the *spt16* H3(K4)R mutant, the lack of binding of a chromodomain protein, possibly Chd1, is toxic in the presence of the defective yFACT complex. The Eaf3 chromodomain protein has been shown to bind methylated H3 K36 (Carrozza *et al*, 2005; Joshi and Struhl, 2005; Keogh *et al*, 2005). Eaf3 is present in two complexes, NuA4 and Rpd3S, and thus it is possible that the absence of one of these complexes and their associated enzymatic activities suppresses the growth defects of the *spt16* and *pob3* mutant strains. It has also been shown that K36 methylation by Set2 recruits the Rpd3S histone deacetylase complex to the 3′ portions of coding regions. In *set2* or H3(K36R) mutants, there could be a redistribution of Rpd3S from coding regions to promoters, and thus the effect of *set2* on TBP binding could be indirect. Further work will be needed to decipher the molecular mechanisms of how loss of H3 K36 methylation suppresses yFACT mutants.

Materials and methods

Yeast strains are listed in Supplementary Table S1. Cells were grown in YPD medium (Sherman, 1991) at 30°C, except where other temperatures are noted, or in synthetic complete medium (Sherman, 1991) with 2% glucose and supplemented with adenine, uracil and amino acids, as appropriate, to select for plasmids. For the galactose induction experiments, cells were grown at 25°C in YP medium supplemented with 2% raffinose to mid log, shifted to 30°C for growth for 2 h, and then galactose was added to a final concentration of 2%. Plasmids are listed in Supplementary Table S2. The histone mutations were generated by site-directed mutagenesis, and the sequences verified.

RNA levels were determined with S1 nuclease protection assays as described (Bhoite and Stillman, 1998) using probes listed in Supplementary Table S3. Chromatin immunoprecipitations were performed as described (Ausubel *et al*, 1987) using the 8WG16 monoclonal antibody against the pol II C-terminal repeat, and a polyclonal anti-TBP sera generously provided by Tony Weil, (Schroeder *et al*, 2000). Real-time PCR and calculations were performed as described (Eriksson *et al*, 2004), using the ORF-free chromosome I region (Mason and Struhl, 2005) as the internal control.

Supplementary data

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

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