The requirements for COMPASS and Paf1 in transcriptional silencing and methylation of histone H3 in *Saccharomyces cerevisiae*

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The Set1-containing complex, COMPASS, methylates histone H3 on lysine 4 (K4) in *Saccharomyces cerevisiae*. Despite a preferential association of K4-trimethylated histone H3 with regions of the genome that are transcribed by RNA polymerase II, transcriptional silencing is one of the few cases in *S. cerevisiae* where histone-methylation defects have a clear effect on gene expression. To better understand the role of COMPASS in transcriptional silencing, we have determined the members of COMPASS that are required for gene silencing at the ribosomal DNA locus (rDNA), a telomere, and the silent mating loci (*HM*). Our findings indicate that most members of COMPASS are required for silencing at the rDNA and telomere, while none are required for silencing of endogenous genes at the *HM* loci. To complement gene-expression analysis, we determined the members of COMPASS that are required for methylation of histone H3. While most are required for trimethylation, cells lacking certain COMPASS proteins maintain reduced levels of K4-mono- and dimethylated H3, suggesting that some members of COMPASS have redundant function and the possibility of more than one complex proficient in K4 methylation of histone H3. Finally, we show data that implicate the Paf1 elongation complex in recruitment of COMPASS to silent chromatin.
Transcriptional silencing is a form of repression of RNA polymerase II (Pol II)-transcribed genes that occurs in a regional, rather than promoter-specific manner. In *Saccharomyces cerevisiae*, three genomic regions where transcriptional silencing occurs have been identified, the homothallic mating (*HM*) loci, telomeres, and the ribosomal DNA locus (rDNA) (reviewed in (Rusche et al. 2003)). Silencing at each of these genomic regions requires Sir2, an NAD-dependent histone deacetylase. At the *HM* loci and telomeres, Sir2 functions as a member of the Silent Information Regulator (Sir) complex, consisting of Sir2, Sir3 and Sir4. In many cases, the requirements for transcriptional silencing at the rDNA are different from those at the *HM* loci and telomeres. While Sir2 is required for rDNA silencing, Sir3 and Sir4 are not (Bryk et al. 1997; Fritze et al. 1997; Smith and Boeke 1997). At the rDNA, Sir2 functions as a member of the RENT complex that also contains Net1, a protein required for nucleolar stability, and Cdc14, a protein phosphatase. RENT associates with the rDNA, but does not associate with the *HM* loci or telomeres (Straight et al. 1999).

Post-translationally modified histones are required for transcriptional silencing in *S. cerevisiae* (reviewed in (Jenuwein and Allis 2001; Strahl and Allis 2000)). Deacetylated histones play a direct role in transcriptional silencing. Sir2 deacetylates lysine residues in the tails of histones H3 and H4 creating a chromatin domain that is favorable for the binding of Sir3 and Sir4 (reviewed in (Rusche et al. 2003)) and refractory to the function of Pol II (Chen and Widom 2005). K4-methylated histone H3 is also important for gene silencing in *S. cerevisiae*. Set1 is a histone methyltransferase that is required for the mono-, di- and trimethylation of lysine 4 (K4) of histone H3 in *S. cerevisiae* (Briggs et al. 2001; Krogan et al. 2002; Nagy et al. 2002; Roguev et al.)
Set1 belongs to a multi-subunit complex called COMPASS, whose members include Set1, Bre2, Sdc1, Shg1, Spp1, Swd1, Swd2, and Swd3 (Krogan et al. 2002; Nagy et al. 2002; Roguev et al. 2001). Of the eight proteins in COMPASS, only Swd2, a WD-40 motif-containing protein, is essential for viability as it is required for correct termination of Pol II transcription (Cheng et al. 2004; Dichtl et al. 2004). The seven remaining members of COMPASS are not essential meaning that cells lacking one or more of the seven nonessential members of COMPASS are viable, as are strains that lack K4-methylated histone H3.

Cells lacking Set1 do not form a functional COMPASS complex (Roguev et al. 2001) and yet have few phenotypes. One of the most prominent defects of set1Δ cells is the inability to silence Pol II-transcribed genes at telomeres and the rDNA (Bryk et al. 2002; Krogan et al. 2002; Nagy et al. 2002; Nislow et al. 1997). Several studies have examined the requirement for COMPASS members in silencing at telomeres (Krogan et al. 2002; Miller et al. 2001; Nagy et al. 2002; Nislow et al. 1997; Schneider et al. 2005). However, conflicting results from these studies have left the question of which COMPASS proteins are required for silencing at telomeres unresolved. The roles of the COMPASS members other than Set1 in transcriptional silencing at the rDNA or of endogenous genes at the HM loci have not yet been determined.

High levels of K4-trimethylated histone H3 are associated with genes that have been recently transcribed by Pol II (Bernstein et al. 2002; Boa et al. 2003; Santos-Rosa et al. 2002). Recruitment of COMPASS to actively transcribed genes occurs through interactions with the Pol II-elongation complex Paf1C (Krogan et al. 2003; Ng et al. 2003b). In addition, Paf1C is required for ubiquitylation of histone H2B by Ubc2.
(Rad6), a modification that is a prerequisite for methylation of histone H3 present at the 5' end of actively transcribed genes by COMPASS (Briggs et al. 2002; Ng et al. 2003a; Wood et al. 2003). Cells lacking Paf1 or Ubc2 fail to accumulate K4-trimethylated H3 at Pol II-transcribed genes (Dover et al. 2002; Krogan et al. 2003; Ng et al. 2003a; Ng et al. 2003b; Sun and Allis 2002; Wood et al. 2003). Despite a wealth of knowledge about the regulation of K4 methylation of histone H3, the function of K4-methylated histone H3 at actively transcribed genes remains unclear.

A report examining the role of COMPASS in directing methylation of histone H3 after induction of expression of the MET16 gene stated that Set1, Swd1, and Swd3 are required for K4-dimethylated H3, while Set1, Swd1, Swd3, Sdc1, and Spp1 are required for K4-trimethylated H3 (Morillon et al. 2005). Other studies reported that Bre2 is required for K4-trimethylation of histone H3, and that loss of K4-trimethylated H3 in cells lacking Bre2 can be restored partially in the presence of a specific dominant allele of SET1 (Schlichter and Cairns 2005; Schneider et al. 2005). While these and other studies have looked at a subset of COMPASS members in methylation of histone H3, a systematic analysis to determine the role of each of the seven nonessential COMPASS members in K4-mono, di-, or trimethylation of histone H3 has not been reported.

Here, we examine the requirement for individual COMPASS members in transcriptional silencing and K4-mono-, di-, and trimethylation of histone H3. Our data show that with the exception of Shg1, all members of COMPASS are required for transcriptional silencing at the rDNA. In contrast to the rDNA, we found that all members of COMPASS are required for silencing of a Pol II gene at the telomere on the left arm of chromosome VII, while none are required for silencing of endogenous genes.
located at the *HM* loci. Using strains lacking one or more of the genes encoding subunits of COMPASS, we provide evidence that COMPASS complexes lacking one or more subunits maintain the ability to methylate histone H3. These findings indicate a redundancy in the roles of several COMPASS members in the mono- and dimethylation of histone H3 on K4. Similar to the requirement for Paf1C in targeting COMPASS to genes transcribed by RNA Pol II, Paf1 is required for gene silencing and wild-type levels of K4-methylated histone H3 at the rDNA, suggesting a possible direct role for K4-methylated H3 in gene silencing at the rDNA.

**MATERIALS AND METHODS**

**Media:** Standard yeast media were prepared as described (Rose *et al.* 1990). YPADT is YPD media supplemented with L-tryptophan (20 mg/L) and adenine sulfate (20 mg/L). Synthetic complete (SC) media and SC containing 1 mg/L 5-fluoroorotic acid (SC + 5-FOA) (Boeke *et al.* 1987) was used to measure the level of expression of a *URA3* gene at the telomere on the left arm of chromosome VII.

**Yeast strains:** *S. cerevisiae* strains are shown in Table 1. MBY1198, MBY1217 and MBY1238 have been described previously (Bryk *et al.* 2002). All other strains were made for this study. A set of six strains, each with one of the genes encoding a COMPASS protein deleted and replaced with the *KANMX4* gene from pRS400 (Brachmann *et al.* 1998) was made by PCR-mediated gene disruption (Schneider *et al.* 1995) in MBY1198. A *shg1Δ::KANMX4 spp1Δ::LEU2* double mutant was made by PCR-mediated gene disruption (Schneider *et al.* 1995) in MBY1666 (See Table 1). Other multiple COMPASS gene deletion mutants were made by genetic crosses. The
URA3 gene was integrated into the ADH4 locus at the left end of chromosome VII (URA3-TEL-VII) in MBY1198 and the COMPASS gene deletion strains using plasmid ADH4UCA-IV kindly provided by Dan Gottschling (GOTTSCHLING et al. 1990). PAF1 was deleted and replaced with KANMX4 from pRS400 or URA3 from pRS406 (BRACHMANN et al. 1998), and SIR2 was deleted and replaced with KANMX4 from pRS400 by PCR-mediated gene disruption (SCHNEIDER et al. 1995). Strains containing gene deletions or insertions were checked by restriction digest of PCR-amplified genomic DNA and genetic crosses to verify Mendelian segregation of KANMX4, LEU2, or URA3 marking the gene deletions and URA3 marking the telomere on the left end of chromosome VII.

Plate assay for expression of the telomeric URA3 gene: Cultures containing 5 mL of YPADT medium were seeded with wild type or COMPASS gene deletion cells containing URA3-TEL-VII and grown to saturation. Ten-fold serial dilutions of each culture were made in sterile milliQ water and 5 µL of each dilution was spotted onto SC and SC + 5-FOA agar. Plates were photographed after 2-3 days of incubation at 30°C.

Western blot analysis: Cells grown to early-log phase (1-2 x 10^7 cells/mL) in 50 mL YPADT medium were washed with milliQ water and resuspended in 0.25 mL RIPA buffer (HARLOW and LANE 1999) containing 1% SDS and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1.67 µg/mL aprotonin, 1.67 µg/mL pepstatin, 0.33 µg/mL leupeptin, 0.4 µg/mL bestatin). Cells were disrupted at 4°C with acid-washed glass beads in a mini-bead-beater (Biospec Products). Lysates were incubated on ice for 30 min and clarified by centrifugation at 12,000 rpm at 4°C for 30 min. Proteins from clarified whole cell extracts (4-100 µg) were separated on 15% SDS-polyacrylamide gels, transferred to PVDF membrane, and probed with α-histone H3 (Abcam; 1:2,500),
\( \alpha \)-K4-monomethyl H3 (Abcam; 1:125 & 1:2,000), \( \alpha \)-K4-dimethyl H3 (Upstate Cell Signaling; 1:5,000) or \( \alpha \)-K4-trimethyl H3 (Abcam; 1:5,000). Antibody binding was detected using HRP-conjugated \( \alpha \)-rabbit secondary antibodies (Promega; 1:2,000) and an Immun-Star chemiluminescence kit (BioRad). Western blots were quantified on a Fujifilm LAS-3000 image analyzer using ImageGauge and IJAR software.

**Northern blot analysis:** Total RNA was isolated from yeast cells as described previously (Bryk et al. 1997). Northern analysis was performed as described (Swanson et al. 1991). Strand-specific \( ^{32} \)P-labeled RNA probes were used to detect Ty1his3A1, total Ty1 and PYK1 mRNAs (Curcio and Garfinkel 1992). \( ACT1 \) (+564 to +1,200), \( URA3 \) (+134 to +679), \( a1 \) (+173 to +405) and \( \alpha2 \) (+166 to +523) probes were made by PCR amplification of yeast genomic DNA, and then purified from agarose gels and labeled with \( \alpha \)\(^{-32} \)P-dATP by random priming (Ausubel et al. 1988). Northern blots were quantified on a Molecular Dynamics Storm 860 phosphorimager using ImageQuant software.

**Chromatin immunoprecipitation/slot-blot analysis:** Chromatin immunoprecipitations (ChIP) were performed according to Bryk et al., 2002 with some modifications. Cultures were grown to mid-log phase, crosslinked chromatin was prepared and sheared by sonication at 4°C to an average size of 500 base pairs with twelve 20-second pulses using a Branson Sonifier 250 at a 1.5 power setting, 100% duty cycle. Sonicated chromatin was incubated with 6 \( \mu \)L of \( \alpha \)-K4-monomethyl H3 (Abcam), 15 \( \mu \)L of \( \alpha \)-K4-dimethyl H3 (Upstate Cell Signaling) or 5 \( \mu \)L of \( \alpha \)-K4-trimethyl H3 (Abcam) for 16 hours at 4°C. Immune complexes were isolated after incubation at 4°C for 2 hours with Protein A or Protein G sepharose beads (Amersham Biosciences). Formaldehyde crosslinks
were reversed by incubation of the immunoprecipitated or input chromatin samples for 12-16 hours at 65°C. Next, the chromatin was treated with proteinase K, and the DNA was purified by phenol and chloroform extraction, precipitated with ethanol, and analyzed by slot blot. The $^{32}$P-labeled probes specific for the non-transcribed spacer of the rDNA locus (rDNA NTS) and the telomere on the left arm of chromosome VI (TelVIR) were described previously (BRYK et al. 2002). Percent immunoprecipitation (%IP) is the ratio of the signal from immunoprecipitated DNA to the signal from input DNA. The %IP was determined by quantification of the blots on a Storm 860 phosphorimager (Molecular Dynamics) using ImageQuant software. The %IP values for the wild-type and spp1Δ extracts were corrected for background by subtracting the %IP value obtained for set1Δ cells, where no H3 K4-methylation occurs.

Oligonucleotides: The sequences of oligonucleotides are available upon request.

RESULTS

Several COMPASS members are required for silencing in the rDNA: Previous work has shown that SET1 is required for gene silencing at the rDNA in S. cerevisiae (BRIGGS et al. 2001; BRYK et al. 2002). To determine the requirement for other members of COMPASS in rDNA silencing, a set of strains with each lacking one of the seven nonessential genes encoding a COMPASS protein was constructed. Each of these strains contains a single copy of a Pol II-transcribed Ty1his3AI element in the rDNA (rDNA-Ty1his3AI element) (BRYK et al. 1997). The level of mRNA from the rDNA-Ty1his3AI element in total RNA isolated from the seven COMPASS deletion strains and the wild-type strain MBY1198 (Table 1) was measured to determine if the deleted
COMPASS gene was required to silence Pol II transcription in the rDNA (Figure 1). The low level of rDNA-Ty1his3Al transcript in total RNA from wild-type cells (WT) reflects transcriptional silencing (Figure 1, top panel). We measured the level of ACT1 mRNA as a normalization control. In the COMPASS deletion strains, with the exception of the shg1Δ mutant, the level of the rDNA-Ty1his3Al transcript relative to ACT1 mRNA was increased 2.5- to 5.3-fold above that of the wild-type strain. In contrast, the ratio of the rDNA-Ty1his3Al:ACT1 mRNA in shg1Δ cells was similar to the level in wild-type cells (See legend for Figure 1). Haploid S. cerevisiae strains contain approximately 30 endogenous Ty1 elements (WILKE et al. 1992). As a control to show that the transcriptional defects in the COMPASS deletion mutants were specific to the rDNA, we monitored steady-state mRNA from the endogenous Ty1 elements using a Ty1-specific probe (Figure 1, middle panel). Importantly, the level of total Ty1 mRNA was nearly equivalent in the COMPASS deletion mutants and the wild-type strain, indicating that the COMPASS proteins are required for silencing of the Ty1his3Al element in the rDNA and do not regulate expression of Ty1 elements located outside of the rDNA. In summary, these results indicate that SET1, BRE2, SDC1, SPP1, SWD1, and SWD3 are required for transcriptional silencing of the rDNA-Ty1his3Al element, and that SHG1 is not required for rDNA silencing. These findings are consistent with the results of quantitative transposition assays that measure the level of expression of the rDNA-Ty1his3Al element (data not shown).

All COMPASS members are required for silencing of a URA3 gene integrated at a telomere: Several studies have analyzed the requirement for COMPASS members in
telomeric silencing (Krogh et al. 2002; Miller et al. 2001; Nagy et al. 2002; Nislow et al. 1997; Schneider et al. 2005). Despite the use of similar strains and plate-growth assays, some of the results are contradictory. To resolve these discrepancies, in addition to plate assays, we used a more direct method to determine the genetic requirements for telomeric silencing by measuring the level of RNA transcript from a Pol II-transcribed gene inserted at a telomere. We integrated the URA3 gene at a telomere in a wild-type strain and the COMPASS deletion strains (Materials and Methods; Table 1), and performed Northern analysis to evaluate the role of COMPASS members in telomeric silencing by comparing the levels of URA3 mRNA in the wild type and COMPASS deletion strains (Figure 2A). The low level of URA3 transcript in total RNA from the wild-type strain indicates that the URA3 gene was silenced at the telomere. We measured the level of ACT1 mRNA as a normalization control. In the COMPASS deletion mutants, the ratio of URA3:ACT1 mRNA was increased 3.7- to 9.7-fold above that of the wild-type strain (See legend for Figure 2).

To complement the Northern analysis, plate-growth assays were performed (Figure 2B). The chemical 5-fluoroorotic acid (5-FOA) is converted by the URA3 gene product to the toxic analog 5-fluorouracil. Growth of cells on media containing 5-FOA (SC + 5-FOA) indicates that the URA3 gene is silenced at the telomere, whereas the lack of growth on 5-FOA media means that URA3 is expressed and that telomeric silencing is defective. In plate assays using wild-type cells, the level of expression of the telomeric URA3 gene was extremely low, as evidenced by robust growth on SC + 5-FOA (Figure 2B). In contrast, in COMPASS deletion mutant cells, the URA3 gene was expressed at higher levels, leading to a 10,000-fold reduction in growth on SC + 5-FOA.
in the \( set1\Delta \), \( sdc1\Delta \), \( swd1\Delta \), and \( swd3\Delta \) mutants, between a 100- to 1000-fold reduction in the \( \text{bre2}\Delta \) mutant and a 10- to 100-fold reduction in the \( \text{spp1}\Delta \) mutant. In the \( \text{shg1}\Delta \) cells, a 5- to 10-fold reduction in growth was observed on SC + 5-FOA (Figure 2B), a result that is consistent with the Northern analysis showing that deletion of \( \text{SHG1} \) has the smallest effect of all COMPASS deletion mutants on the level of mRNA from the telomeric \( \text{URA3} \) gene (Figure 2A). Our findings using Northern blotting and plate assays demonstrate that all members of COMPASS are required for transcriptional silencing of the \( \text{URA3} \) gene when located at the telomere. However, there is assay-dependent variability in the degree of the loss-of-silencing phenotype observed in the COMPASS deletion mutants.

**COMPASS is not required for silencing of endogenous genes at the \( \text{HM} \) loci:**

Mating-type-specific genes present at the \( \text{HM} \) loci are not expressed in wild-type cells. Thus, the \( \text{a1} \) gene is silenced when present at \( \text{HMR} \) (referred to as the \( \text{HMRa1} \) gene), but is expressed if present at the \( \text{MAT} \) locus (i.e. in a \( \text{MATa} \) strain). Likewise, the \( \alpha2 \) gene is silenced when present at \( \text{HML} \), but is expressed when located at the \( \text{MAT} \) locus in \( \text{MAT}\alpha \) cells. To examine the role of COMPASS in transcriptional silencing at \( \text{HMR} \), we measured the level of \( \text{HMRa1} \) transcript in wild type \( \text{MAT}\alpha \) cells (WT \( \text{MAT}\alpha \)) and COMPASS deletion \( \text{MAT}\alpha \) cells (\( \text{set1}\Delta \text{MAT}\alpha \), \( \text{bre2}\Delta \text{MAT}\alpha \), \( \text{sdc1}\Delta \text{MAT}\alpha \), etc) by Northern analysis (Figure 3A). The lack of detectable \( \text{HMRa1} \) transcript in total RNA from wild-type \( \text{MAT}\alpha \) cells indicates that the \( \text{HMRa1} \) gene was silenced. Similarly, \( \text{HMRa1} \) transcript was not detected in total RNA from the COMPASS deletion mutants, indicating that transcriptional silencing of \( \text{HMRa1} \) does not require COMPASS. As
controls, total RNA from a \textit{MATa} wild-type strain where the \textit{a1} gene is present at the \textit{MAT} locus and a \textit{MAT\alpha\,sir2\Delta} strain in which deletion of the gene encoding the silencing factor \textit{SIR2} causes loss of silencing at the \textit{HMR\alpha1} locus were included on the blot. In both wild-type \textit{MATa} cells (WT \textit{MATa}) and \textit{MAT\alpha\,sir2\Delta} cells (\textit{sir2\Delta\,MAT\alpha}), \textit{a1} transcripts were detected (Figure 3A), indicating that the \textit{a1} mRNA from the \textit{MAT} or \textit{HMR} locus can be detected by Northern analysis if present. Similar results were obtained when examining \textit{HML\alpha2} RNA from wild type \textit{MATa} and \textit{set1\Delta\,MATa} cells. No \textit{HML\alpha2} transcript was present in total RNA from wild type or \textit{set1\Delta} cells, however, \textit{MAT\alpha2} transcripts were detected in total RNA from wild type \textit{MAT\alpha} (WT \textit{MAT\alpha}) cells and \textit{sir2\Delta\,MATa} cells (Figure 3B). Together, these data indicate that COMPASS is not required for transcriptional silencing of endogenous genes present at the \textit{HM} loci.

**Overlapping requirements for COMPASS proteins in methylation of histone H3 on K4:** To determine the requirement for COMPASS members in the methylation of histone H3 on K4, we analyzed whole cell extracts isolated from wild-type cells and COMPASS deletion mutants by Western blotting. Total histone H3 was measured using antisera that recognizes the C-terminus of histone H3, and K4-monomethylated histone H3, K4-dimethylated histone H3, and K4-trimethylated histone H3 were measured using antisera that recognize one of the three forms of K4-methylated histone H3 (Figure 4A; Materials and Methods). The data indicate that Set1, Swd1, and Swd3 are required for mono-, di-, and trimethylation of histone H3 on K4, and that Bre2 and Sdc1 are required for trimethylation of histone H3 on K4. While the levels of K4-mon- and K4-dimethylated H3 in extracts from \textit{bre2\Delta} or \textit{sdc1\Delta} cells are lower than the levels
found in extracts from wild-type cells, the analysis revealed that K4 mono- and
dimethylation of histone H3 can occur in the absence of BRE2 or SDC1. All three forms
of K4-methylated histone H3 were present in spp1Δ cells and in shg1Δ cells, albeit at
levels lower than in wild-type cells. Consistent with the small effects of SHG1 on gene
silencing at the rDNA and telomere (Figures 1 and 2), extracts from shg1Δ cells
contained the highest levels of K4-mono-, di-, and trimethylated H3 of all the COMPASS
deletion mutants.

The analysis shown in Figure 4A suggests that a core set of COMPASS
members, including Set1, Swd1, and Swd3, is essential for the mono- and dimethylation
of histone H3 on K4, and that these core proteins, along with Bre2 and Sdc1, are
required for trimethylation of histone H3 on K4. Further, while K4-mono-, di-, and
trimethylated forms of histone H3 were detected in extracts from cells lacking Spp1, the
level of K4-trimethylated H3 was less than 10% of the level detected in wild-type
extracts, suggesting an important role for Spp1 in trimethylation of H3 on K4. To further
understand the requirements for Bre2, Sdc1, Spp1, and Shg1 in the methylation of
histone H3, we constructed and characterized double and triple gene-deletion mutants
(Figure 4B). Western analysis on extracts from cells lacking both BRE2 and SDC1
showed that these cells have no detectable K4-trimethylated histone H3 and reduced
levels of K4-mono- and dimethylated histone H3. The levels of K4 mono- and
dimethylated histone H3 in the bre2Δ sdc1Δ double mutant are similar to those in the
 corresponding single deletion mutants (Figure 4A) suggesting that BRE2 and SDC1 are
redundant with each other with respect to their role in methylation of histone H3. These
data indicate that, in cells with an otherwise complete COMPASS complex, Bre2 and Sdc1 are not absolutely required for K4 mono- or dimethylation of H3.

To understand the contribution of Shg1 to the histone H3 methylation, we analyzed extracts from multiple deletion mutant cells lacking $\text{BRE2}$ and $\text{SHG1}$ ($\text{bre2}\Delta \text{shg1}\Delta$), $\text{SDC1}$ and $\text{SHG1}$ ($\text{sdc1}\Delta \text{shg1}\Delta$), or $\text{BRE2}$, $\text{SDC1}$, and $\text{SHG1}$ ($\text{bre2}\Delta \text{sdc1}\Delta \text{shg1}\Delta$) (Figure 4B). The levels of K4-methylated H3 were reduced to a similar degree in extracts from these multiple deletion mutants with the levels of K4-monomethylated H3 at less than 10% of wild type and the levels of K4-dimethylated histone H3 at less than 1% of wild type. Interestingly, cells lacking $\text{SHG1}$ ($\text{shg1}\Delta$) maintain levels of K4 mono- and dimethylated histone H3 that are not significantly different from the levels in wild-type cells (Figure 4A) and cells lacking Bre2 and/or Sdc1 maintain higher levels of K4-mono- and dimethylated H3 when Shg1 is present (Figure 4A and B). Thus, these data reveal that in the absence of Bre2 and/or Sdc1, Shg1 is important for the function and/or stability of the COMPASS complex.

Next, we analyzed the role of Spp1 in COMPASS by measuring the levels of K4-methylated H3 in cells lacking $\text{BRE2}$ and $\text{SPP1}$ ($\text{bre2}\Delta \text{spp1}\Delta$), $\text{SDC1}$ and $\text{SPP1}$ ($\text{sdc1}\Delta \text{spp1}\Delta$), or $\text{SHG1}$ and $\text{SPP1}$ ($\text{shg1}\Delta \text{spp1}\Delta$) (Figure 4B). Our data show that extracts from cells lacking Spp1 and Bre2 or Spp1 and Sdc1 were devoid of K4-methylated histone H3 indicating that H3 K4-mono- and K4-dimethylation requires either Spp1 and Bre2 or Spp1 and Sdc1. Cells lacking both Shg1 and Spp1 contained no K4-trimethylated histone H3 and reduced levels of K4-mono- and dimethylated H3 compared to the single deletion mutants (See Figure 4A). These data reveal that neither Spp1 nor Shg1 is absolutely required for trimethylation of K4 of histone H3.
However, the lack of K4-trimethylated H3 and reduced K4-mono- and dimethylated H3 in the \textit{spp1}\Delta \textit{shg1}\Delta double mutant indicates that Spp1 and Shg1 make independent contributions to the function or stability of COMPASS. Consistent with the silencing defects of the single \textit{spp1}\Delta mutant and the H3-methylation defects of the double mutant, using Northern analysis, we determined that the double \textit{shg1}\Delta \textit{spp1}\Delta mutant is defective for rDNA silencing (data not shown).

**Bulk K4-methylated histone H3 in \textit{spp1}\Delta cells mirrors the levels associated with the rDNA and a telomere:** To determine if the levels of K4-methylated histone H3 measured in WCEs reflected the levels present at silent loci, we performed chromatin immunoprecipitation analyses (ChIP) to measure K4-methylated H3 at the rDNA non-transcribed spacer (rDNA NTS) and the telomere at the left arm of chromosome VI (\textit{TelVIR}) in wild-type, \textit{spp1}\Delta, and \textit{set1}\Delta cells. Input and immunoprecipitated DNA was analyzed using a slot blot to determine the fraction of rDNA NTS or \textit{TelVIR} DNA associated with K4-methylated histone H3 (Materials and Methods; Figure 5A). Consistent with the reduced levels of bulk K4-methylated H3 in \textit{spp1}\Delta cells, the association of rDNA NTS and \textit{TelVIR} with the three forms of K4-methylated H3 was lower in \textit{spp1}\Delta cells than in wild-type cells. To facilitate a comparison of ChIP and Western data, in Figure 5B, the ratios of the level of rDNA NTS or \textit{TelVIR} associated with K4-methylated H3 as measured by ChIP (Figure 5A) are represented graphically alongside the ratios of K4-methylated H3 in \textit{spp1}\Delta cells relative to wild-type cells measured by Western analysis (Figure 4A). This representation shows that in \textit{spp1}\Delta
cells the levels of bulk K4-methylated H3 are reduced and correspondingly the levels of K4-methylated H3 are lower at the rDNA NTS and TelVIR.

**PAF1 is required for rDNA silencing:** Studies have implicated the Paf1 complex in methylation of histone H3 on K4 and silencing at telomeres (Krogan *et al.* 2003; Ng *et al.* 2003b). Because *PAF1* was reported to be required for methylation of histone H3 on K4, we reasoned that cells lacking *PAF1* would exhibit defects in rDNA silencing. Total RNA from wild type and *paf1*Δ cells carrying a *URA3* marker (*mURA3*) inside the rDNA or outside the rDNA (Smith and Boeke 1997) was analyzed by Northern to determine if *PAF1* is required for rDNA silencing (Figure 6A). The data indicate that the ratio of the *mURA3* (inside rDNA):*ACT1* mRNA was increased 2.9-fold in the *paf1*Δ cells compared to wild-type cells, consistent with a defect in rDNA silencing. The ratio of *mURA3* (outside rDNA):*ACT1* mRNA was 1.1 in *paf1*Δ cells, similar to wild-type cells. Thus, these data show that *PAF1* is required for transcriptional silencing at the rDNA.

The levels of K4-methylated histone H3 at the rDNA were measured by ChIP in wild type, *paf1*Δ, and *set1*Δ cells. Analysis of immunoprecipitated DNA showed that the levels of K4-di- and trimethylated histone H3 at the rDNA NTS in *paf1*Δ cells were reduced relative to those in wild-type cells (Figure 6B). The level of K4-dimethylated H3 at the rDNA in the *paf1*Δ strain was 37% of that in wild type, while the level of K4-trimethylated H3 was 18% of that in wild type. The presence of K4-methylated histone H3 in the *paf1*Δ strain is consistent with the results of Schlichter and Cairns, who also detected K4-methylated forms of histone H3 in WCEs prepared from *paf1*Δ strains (Schlichter and Cairns 2005).
DISCUSSION

In this study, we examined the requirements for individual members of the COMPASS complex in methylation of histone H3 and in transcriptional silencing at the rDNA locus, telomeres, and the silent mating loci in *S. cerevisiae*. We found that most members of the COMPASS complex with the exception of Shg1 are required for transcriptional silencing at the rDNA (Figure 1). In contrast to the rDNA, we found that all members of the COMPASS complex are required for silencing of a gene at a telomere (Figure 2), and that none were required for silencing of the endogenous *a1* and *α2* genes at *HMR* and *HML*, respectively (Figure 3). Given that the levels of all three forms of K4-methylated histone H3 are altered to different extents in the COMPASS deletion mutants (Figure 4), we cannot assign which forms of K4-methylated H3 are required for gene silencing. However, it is clear that loss of and reduction of K4-mono-, di- and trimethylated H3 are associated with loss of silencing at the rDNA and telomeres. The results of studies with multiple deletion mutants suggest that some members of COMPASS have redundant function (Figure 4B), hinting at the possibility of functional COMPASS subcomplexes *in vivo*. In addition, there is a requirement for Paf1 in rDNA silencing (Figure 6A). Considering that Paf1 is required for targeting of COMPASS to actively transcribed genes (Krogan *et al.* 2003; Ng *et al.* 2003b) and reduced levels of K4-di and trimethylated H3 are present at the rDNA in *paf1Δ* cells (Figure 6B), one possibility is that Paf1 recruits COMPASS to the rDNA and other silent loci.
We examined the role of COMPASS in silencing at the rDNA. Our data suggest that, with the exception of Shg1, all members of COMPASS are required for rDNA silencing. Our results show that rDNA silencing occurs in \textit{shg1Δ} cells where the level of K4-trimethylated H3 is about 50% of wild type, suggesting that silent chromatin at the rDNA is tolerant of ~2-fold changes in the level of K4-trimethylated H3. Given that the levels of all three forms of K4-methylated histone H3 are reduced in the COMPASS deletion mutants found to be required for rDNA silencing (Figure 4), our data cannot confirm or refute a previous report that only K4-trimethylated H3 is required for rDNA silencing (\textit{FINGERMAN et al.} 2005).

We examined the requirement for COMPASS in silencing at the telomere on the left arm of chromosome VII using Northern analysis and plate growth assays. Our results show that all COMPASS members are required for telomeric silencing (Figure 2). These data are in agreement with reports of Krogan \textit{et al.} and Schneider \textit{et al.} who examined the role of COMPASS members in telomeric silencing previously, although Shg1 was not tested (\textit{KROGAN et al.} 2002; \textit{SCHNEIDER et al.} 2005). Similar to these previous studies, our plate growth assays showed partial loss of silencing in \textit{bre2Δ} cells and \textit{spp1Δ} cells. Our Northern analysis indicated a clear requirement for Bre2 and Spp1 in telomeric silencing, as \textit{URA3} transcript levels in total RNA from \textit{bre2Δ} and \textit{spp1Δ} cells were 6.7- and 4.2-fold greater than wild type, respectively (Figure 2). Further, our data reveal that \textit{shg1Δ} mutants and \textit{spp1Δ} mutants, which are the least affected in the telomeric silencing assays (Figure 2), have levels of K4-mono, di-, and trimethylated H3 that are closest to wild type (Figure 4A). ChIP experiments with a \textit{spp1Δ} mutant (Figure 5) and a \textit{shg1Δ} mutant (data not shown) show that the levels of
K4-methylated H3 at TelVIR reflect the levels in WCEs. Together, these data suggest that in these mutants, the level of K4-methylated H3 is related to the degree of the loss of telomeric silencing phenotype.

The role for Shg1 in transcriptional silencing at telomeres is intriguing, given that Shg1 is not required for silencing at the rDNA. We conclude that while the subtle reduction in levels of K4-methylated H3 in the shg1Δ mutant does not disrupt rDNA silencing, it is sufficient to derepress Pol II gene transcription at telomeres. This is consistent with the suggestion that telomeric silencing is highly sensitive to changes in the levels of factors that are involved in gene silencing in S. cerevisiae (APARICIO et al. 1991). Silencing at telomeres and the HM loci require several factors, including Sir3 and histone H4. Mutations in SIR3 suppress mutations in the gene encoding histone H4 (HHF2) and thereby restore silencing at the silent mating-type locus HML but not at telomeres (APARICIO et al. 1991). Accordingly, telomeric silencing may be more susceptible to changes in the levels of K4-methylated H3, resulting in a requirement for Shg1 in transcriptional silencing at telomeres, but not at the rDNA.

To address the role of COMPASS in silencing at the HM loci, we measured HMRa1 and HMLα2 gene transcripts in the COMPASS deletion mutants by Northern analysis, which provides a direct readout of endogenous gene expression from the HM loci. Previously it was reported that K4-methylation is required for silencing of Pol II-transcribed genes inserted into the HM loci (FINGERMAN et al. 2005; NISLOW et al. 1997; SANTOS-ROSA et al. 2004). While one study indicated that Set1 is required for repression of a Pol II-transcribed gene only at HML (SANTOS-ROSA et al. 2004), a second study demonstrated that Set1 is required for silencing at HML and HMR
Our analysis indicates that K4-methylated H3 is not required to silence endogenous genes at the $HM$ loci (Figure 3). This finding is consistent with the ability of our set1Δ strains of opposite mating type to mate in genetic crosses (data not shown). We conclude that while K4-methylated H3 may be required to regulate marker genes at the $HM$ loci, it does not regulate endogenous genes at the $HM$ loci.

A number of subunits of the COMPASS complex are dispensable for K4-methylation of histone H3, with requirements for mono- and dimethylation being more relaxed than those for trimethylation. Our work shows that a core set of COMPASS subunits, Set1, Swd1, and Swd3, is essential for all forms of K4-methylated H3. K4-mono- and dimethylation can take place in the absence of any single member of COMPASS other than the core members. However, with the exception of the shg1Δ mutant, the levels of K4-mono- and dimethylated H3 are reduced significantly in the COMPASS deletion mutants compared to wild-type cells. Although the levels of K4-methylated H3 are least affected in the shg1Δ mutant, our studies with multiple deletion mutants reveal that in the absence of Bre2 and/or Sdc1, Shg1 is important for the function and/or stability of the COMPASS complex. The data presented here and in other studies (Morillon et al. 2005; Schlichter and Cairns 2005) suggest that some members of COMPASS share overlapping functions. In our studies using multiple deletion mutants (Figure 5), we found that when SPP1 is deleted there is requirement for Bre2 and Sdc1 for mono- and dimethylation, indicating that together Bre2 and Sdc1 can complement loss of Spp1 in the H3 K4-mono- and dimethylation reactions. Recently, it was suggested that Spp1 and Sdc1 may share similar roles in the distribution of RNA polymerase II along active genes (Morillon et al. 2005). ChIP
studies showed that Pol II accumulates at the 5' end of \textit{MET16} and \textit{RPS11B} genes in \textit{spp1Δ} cells and in \textit{sdc1Δ} cells, further suggesting shared redundant functions for some COMPASS subunits.

Data from our genetic analysis suggest the possibility of at least ten partially functional COMPASS subcomplexes, consisting of core members Set1, Swd1 and Swd3, together with various combinations of other subunits, which are able to methylate K4 of histone H3. Our results using single COMPASS deletion mutants reveal that H3 K4 methylation can occur in the absence of one or more COMPASS proteins. Further, biochemical studies have shown that functional COMPASS complexes lacking one member of COMPASS can be isolated from single COMPASS gene deletion mutants (\textit{SCHNEIDER et al.} 2005). While we do not have proof of the existence of such COMPASS subcomplexes in wild-type cells, the possibility of multiple COMPASS subcomplexes with different functions goes hand-in-hand with genomic-survey studies that show that K4-dimethylated histone H3 is associated with active and inactive genes and is the predominant form of K4-methylated histone H3 in \textit{S. cerevisiae} (\textit{BERNSTEIN et al.} 2002; \textit{SANTOS-ROSA et al.} 2002). Indeed, relaxed requirements to form numerous K4-mono- and dimethylation-competent COMPASS subcomplexes may be a factor in maintaining high levels of K4-dimethylated histone H3 over the genome, and lends support to the speculation that the methylation activity of COMPASS is regulated by the availability of COMPASS proteins. Biochemical analysis to isolate COMPASS subcomplexes from wild-type cells and ChIP analysis to measure the levels of K4-methylated H3 at several transcriptionally active and inactive genomic regions in cells
lacking one or more COMPASS members will help us determine if COMPASS subcomplexes exist and function in vivo.

**Acknowledgments:** The authors greatly appreciate the input and contributions of Fred Winston, in whose lab this work was initiated. We wish to acknowledge Cristine Heaps for access to a Fujifilm LAS-3000 image detection system, Dan Gottschling for reagents, Andrea Fuller for assisting with the construction of a paf1Δ strain, J. Ruth German and Christina Zelasko from the NSF-funded REU program in the Department of Biochemistry at Texas A&M University (NSF DBI-0139246), and Jessica Kilgore for early work on telomeric silencing and Western analysis. This research was supported by American Heart Association (Beginning Grant-in-Aid 0365004Y), American Cancer Society (RSG-04-049-01-GMC), and the National Institutes of Health (GM070930).

**LITERATURE CITED**


### TABLE 1. Yeast strains

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MBY1979  MBY1792 paf1Δ::KANMX4
**FIGURE LEGENDS**

**Figure 1. Several COMPASS members are required for rDNA silencing.** Total RNA from wild-type cells and COMPASS deletion cells that each contain a single Ty1his3Al element in the rDNA was probed with a $^{32}$P-labeled HIS3 RNA probe to detect rDNA-Ty1his3Al message (upper panel), a Ty1 RNA probe to detect total Ty1 message (middle panel), or an ACT1 probe as a loading control (lower panel). Transcript levels for Ty1his3Al and total Ty1 were normalized to the ACT1 loading control. The average values of rDNA-Ty1his3Al:ACT1 and total Ty1:ACT1 mRNA for each mutant relative to the ratio in WT cells are shown on the figure. The average values of rDNA-Ty1his3Al:ACT1 (+/- SE; n=3 or 4) were set1Δ:WT 2.5 (+/-0.1); bre2Δ:WT 4.0 (+/-0.6); sdc1Δ:WT 3.3 (+/-0.5); shg1Δ:WT 1.4 (+/-0.1); spp1Δ:WT 5.3 (+/-1.1); swd1Δ:WT 2.7 (+/-0.4); and swd3Δ:WT 4.4 (+/-1.0). The values of total Ty1:ACT1 mRNA were set1Δ:WT 1.3 (+/-0.2); bre2Δ:WT 1.7 (+/-0.4); sdc1Δ:WT 1.4 (+/-0.3); shg1Δ:WT 1.1 (+/-0.3); spp1Δ:WT 1.2 (+/-0.3); swd1Δ:WT 0.9 (+/-0.2); and swd3Δ:WT 1.4 (+/-0.2).

**Figure 2. A role for COMPASS in silencing at the telomere on the left arm of chromosome VII.** (A) Total RNA from wild type and COMPASS deletion strains that carry URA3 at the telomere on the left arm of chromosome VII (Material and Methods, Table 1) were subject to Northern analysis using radiolabeled DNA probes to measure URA3 or ACT1 mRNA. Blots were quantified and the ratios of URA3 transcript (normalized to ACT1 mRNA) in mutant relative to wild-type cells were determined and are shown below the URA3 panel. The values (+/-SE; n=3 or 4) are: set1Δ:WT 6.0 (+/-...
1.1); bre2Δ:WT 6.7 (+/-0.7); sdc1Δ:WT 6.6 (+/- 0.6); shg1Δ:WT 3.7 (+/-0.1); spp1Δ:WT 4.2 (+/-1.3); swd1Δ:WT 7.4 (+/-0.04) and swd3Δ:WT 9.7 (+/-2.2). (B) Representative plate assay (n=3) indicates a requirement for COMPASS in silencing at a telomere. Growth on SC media verifies equivalent numbers of wild type and COMPASS deletion cells were plated. Growth on SC + 5-FOA indicates silencing of the URA3 gene, since 5-FOA is toxic to cells that express the URA3 gene.

**Figure 3. COMPASS is not required for silencing at the HM loci.** (A) Silencing of the HMRα1 gene does not require COMPASS. Total RNAs from wild type (WT), COMPASS deletion MATα cells (with the a1 gene at HMR), and MATα wild-type cells (with the a1 gene at MAT; WT MATα) were analyzed to monitor levels of a1 transcript. ACT1 mRNA was measured as a loading control. (B) Silencing of the α2 gene does not require Set1. Northern analyses were performed on total RNAs from WT MATα, set1Δ MATα, WT MATα, and sir2Δ MATα cells to examine the requirement for Set1 in silencing of the HMLα2 gene.

**Figure 4. K4-methylated H3 is dependent on several members of the COMPASS complex.** Representative data of Western analysis measuring the levels of K4-methylated H3 in WCE from (A) wild-type cells and single COMPASS deletion mutants and (B) wild-type cells and multiple-deletion mutants using antisera specific for K4-monomethylated H3 (α-mono), K4-dimethylated H3 (α-di), or K4-trimethylated H3 (α-tri). Antisera specific for the C terminus of histone H3 (α-H3) was used to ensure equivalent loading of cell extracts in each lane. The values of the ratio of the levels of K4-
methylated H3 from mutant cells relative to wild-type cells for the blot are shown below each panel. The lack of a value indicates that K4-methylated H3 was not detected in mutant. The data for all the replicates are listed below. The average (+/- SE) when n=3 or the average (range) when n=2 are: K4-monomethylated H3, bre2Δ:WT 0.15(+/-0.04), sdc1Δ:WT 0.16(+/-0.04), shg1Δ:WT 0.80(+/-0.08), spp1Δ:WT 0.44(+/-0.06), bre2Δ sdc1Δ:WT 0.31(+/-0.09), bre2Δ shg1Δ:WT 0.03(+/-0.01), sdc1Δ shg1Δ:WT 0.07(+/-0.03), bre2Δ sdc1Δ shg1Δ:WT 0.05 (+/-0.03), shg1Δ spp1Δ:WT 0.27 (0.10-0.44, n=2); K4-dimethylated H3, bre2Δ:WT 0.08(+/-0.06), sdc1Δ:WT 0.08(+/-0.04), shg1Δ:WT 0.80(+/-0.11), spp1Δ:WT 0.36(+/-0.05), bre2Δ sdc1Δ:WT 0.05(+/-0.01), bre2Δ shg1Δ:WT 0.0035 (0.0026-0.0044, n=2), sdc1Δ shg1Δ:WT 0.0018 (0.0011-0.0026, n=2), bre2Δ sdc1Δ shg1Δ:WT 0.0019 (0.0008-0.0029, n=2), shg1Δ spp1Δ:WT 0.12(+/-0.03); K4-trimethylated H3, shg1Δ:WT 0.47(+/-0.05), spp1Δ:WT 0.05(+/-0.01).

**Figure 5. Reduced levels of K4-methylated H3 are present at silent loci in spp1Δ cells.** (A) ChIP analysis shows that the association of K4-methylated H3 with silent loci is reduced in spp1Δ cells. Immunoprecipitated and input DNA were analyzed by slot blot using a 32P-labeled DNA probe specific for the rDNA non-transcribed spacer region (rDNA NTS) or telomere on the right end of chromosome (TelVIR). Open triangles represent increasing amounts of input DNA used to show linearity of the hybridization. The ratios (spp1Δ/WT) of %IP of the rDNA NTS from two independent experiments are: K4-monomethylated H3, 0.57, 0.48; K4-dimethylated H3, 0.32, 0.33; and K4-trimethylated H3, 0.16, 0.15. The ratios (spp1Δ/WT) of %IP of TelVIR from two independent experiments are: K4-monomethylated H3, 0.63, 0.57; K4-dimethylated H3,
0.52, 0.54; and K4-trimethylated H3, 0.24, 0.24. (B) Graphical representation of the average ratio (spp1\textDelta:WT) of the levels of bulk K4-methylated histone H3 measured by Western analyses (Figure 4A) and the average ratio (spp1\textDelta:WT) of %IP of rDNA NTS and TelVIR associated with K4-methylated histone H3 as measured by ChIP (Figure 5A). Error bars, standard deviation for three independent Westerns and range for two independent ChIP experiments.

**Figure 6. Paf1 is required for rDNA silencing and association of K4-methylated H3 with the rDNA.** (A) RNA from the mURA3 gene inside the rDNA was increased in paf1\textDelta strains. Northern analyses were performed on total RNA from wild type and paf1\textDelta cells that have mURA3 inside the rDNA (left panel) or outside the rDNA at the leu2\textDelta1 locus (right panel). For three independent experiments, the average ratio (paf1\textDelta normalized to wild type) (+/- SE) of mURA3 mRNA (inside rDNA) relative to ACT1 mRNA was 2.9 (+/- 0.2) and of mURA3 mRNA (outside rDNA) to ACT1 mRNA was 1.1 (+/- 0.2). (B) Levels of K4-methylated histone H3 associated with the rDNA NTS were reduced in paf1\textDelta cells. ChIP experiments were performed with formaldehyde-crosslinked extracts from wild type, paf1\textDelta, and set1\textDelta cells using antisera specific for K4-dimethylated H3 (α-di) or K4-trimethylated H3 (α-tri). The average value of %IP for the paf1\textDelta cells relative to the %IP for the wild-type cells (+/-SE) from three independent experiments was 0.37 (+/-0.03) for K4-dimethylated histone H3 and 0.18 (+/-0.04) for K4-trimethylated histone H3.
A

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B

- **SC + 5-FOA**
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  - set1Δ
  - bre2Δ
  - sdc1Δ
  - shg1Δ
  - spp1Δ
  - swd1Δ
  - swd3Δ

- **SC**
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