

Histone H3 Lysine 36 Methylation Antagonizes Silencing in *Saccharomyces cerevisiae* Independently of the Rpd3S Histone Deacetylase Complex

Rachel Tompa and Hiten D. Madhani¹

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-2200

Manuscript received November 2, 2006

Accepted for publication November 22, 2006

ABSTRACT

In yeast, methylation of histone H3 on lysine 36 (H3-K36) is catalyzed by the NSD1 leukemia oncoprotein homolog Set2. The histone deacetylase complex Rpd3S is recruited to chromatin via binding of the chromodomain protein Eaf3 to methylated H3-K36 to prevent erroneous transcription initiation. Here we identify a distinct function for H3-K36 methylation. We used random mutagenesis of histones H3 and H4 followed by a reporter-based screen to identify residues necessary to prevent the ectopic spread of silencing from the silent mating-type locus *HMRa* into flanking euchromatin. Mutations in H3-K36 or deletion of *SET2* caused ectopic silencing of a heterochromatin-adjacent reporter. Transcriptional profiling revealed that telomere-proximal genes are enriched for those that display decreased expression in a *set2Δ* strain. Deletion of *SIR4* rescued the expression defect of 26 of 37 telomere-proximal genes with reduced expression in *set2Δ* cells, implying that H3-K36 methylation prevents the spread of telomeric silencing. Indeed, Sir3 spreads from heterochromatin into neighboring euchromatin in *set2Δ* cells. Furthermore, genetic experiments demonstrated that cells lacking the Rpd3S-specific subunits Eaf3 or Rco1 did not display the anti-silencing phenotype of mutations in *SET2* or H3-K36. Thus, antagonism of silencing is independent of the only known effector of this conserved histone modification.

IN the budding yeast *Saccharomyces cerevisiae*, Sir proteins associate with DNA silencer sequences and spread to form silenced chromatin at telomeres and the silent mating-type loci *HML* and *HMR* (JENUWEIN *et al.* 2001; MOAZED 2001; RUSCHE *et al.* 2002). Unlike the initial nucleation event, the spreading of the Sir complex along chromatin appears to be independent of DNA sequence context (for review, see MOAZED 2001). The spread of a complex containing Sir2, Sir3, and Sir4 along DNA is dependent on the histone deacetylase activity of Sir2 (HOPPE *et al.* 2002; LUO *et al.* 2002; RUSCHE *et al.* 2002). Sir3 and Sir4 have greater affinity for H3 and H4 that are hypoacetylated on their N-terminal tails (CARMEN *et al.* 2002). Thus, Sir2-mediated deacetylation of neighboring nucleosomes may promote recruitment of another Sir complex via a protein–protein interaction between Sir4 and Sir2. Due to this mode of self-propagation, euchromatic genes are silenced when integrated near silencer sequences (SCHNELL and RINE 1986; HUANG *et al.* 1997). While many higher eukaryotes use other components in heterochromatin formation instead of or in addition to Sir proteins, these general principles of sequence-dependent nucleation and sequence-independent propagation are conserved (MOAZED 2001). Since all eukaryotic genomes have euchromatic transcriptionally active regions of

DNA that abut silenced heterochromatin, cells must possess means to prevent heterochromatin from ectopically spreading beyond normal boundaries into neighboring euchromatin.

In several systems, boundary element (BE) sequences are employed to block heterochromatin spread (reviewed in BELL *et al.* 2001; LABRADOR and CORCES 2002). In *S. cerevisiae*, the right boundary element of *HMRa* has been characterized as a tRNA^{Thr} gene (DONZE and KAMAKAKA 2001). Mutations that delete this gene or abrogate its transcriptional activity cause ectopic spread of Sir proteins from *HMRa* into neighboring euchromatin (DONZE and KAMAKAKA 2001). However, deletion of this boundary element results in only limited repression of the nearest euchromatic gene (MENEHINI *et al.* 2003). Recent work has shown that multiple euchromatin-associated factors prevent heterochromatic spread in a process termed “anti-silencing” (KIMURA *et al.* 2002; VAN LEEUWEN *et al.* 2002; SUKA *et al.* 2002; KRISTJUHAN *et al.* 2003; MENEHINI *et al.* 2003; SANTOS-ROSA *et al.* 2004; JAMBUNATHAN *et al.* 2005). An example of one such factor is the histone H2A variant H2A.Z (MENEHINI *et al.* 2003). When the gene coding for H2A.Z (*HTZI*) is deleted, Sir proteins spread from telomeres and the silent mating-type loci into neighboring euchromatin to cause ectopic transcriptional silencing of genes in these regions (MENEHINI *et al.* 2003). Similar functions have been observed for several conserved post-translational modifications of core

¹Corresponding author: Genentech Hall, 600 16th St., Room N372C, San Francisco, CA 94143-2200. E-mail: hiten@biochem.ucsf.edu

histones, including methylation on lysine 79 of H3 (H3-K79) and acetylation on lysine 16 of H4 (H4-K16) (KIMURA *et al.* 2002; VAN LEEUWEN *et al.* 2002; SUKA *et al.* 2002).

In this study, we identify a novel anti-silencing function for methylation on lysine 36 of H3 (H3-K36) in *S. cerevisiae*. This modification is catalyzed by the histone methyltransferase Set2, which has previously been implicated in transcriptional elongation and start site selection (LI *et al.* 2002, 2003; STRAHL *et al.* 2002; KROGAN *et al.* 2003; SCHAFT *et al.* 2003; XIAO *et al.* 2003; CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). We found that H3-K36 is involved in anti-silencing through a reporter-based screen aimed at identifying residues in H3 and H4 that are necessary to prevent the spread of Sir-mediated silencing from *HMRa* into neighboring euchromatin. We show by chromatin immunoprecipitation (ChIP) that in the absence of Set2, Sir proteins spread from *HMRa* and telomeres into neighboring euchromatin. We further demonstrate, using whole-genome transcriptional profiling, that genes near telomeres and *HMRa* are enriched for those dependent on Set2 for their expression. Methylation of K36 represses erroneous transcriptional initiation by recruiting the Rpd3S histone deacetylase complex via the chromodomain of Eaf3 (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). We find that cells lacking Eaf3 or Rco1, two subunits of Rpd3S, do not recapitulate the effects of mutations in *SET2* or H3-K36. Thus, K36 methylation has at least two independent euchromatic functions, antagonism of silencing and transcriptional start site selection, which are effected by two independent mechanisms.

MATERIALS AND METHODS

Yeast strains and screen for histone mutants: Yeast strains used in this study are described in supplemental Table S1 at <http://www.genetics.org/supplemental/>. Strain YM2330 was generated from strain JPY16 described in PARK *et al.* (2002) by a plasmid shuffle to replace pDM9 (*URA3 CEN HHT1-HHF1*) with pJP11 (*LYS2 CEN HHT1-HHF1*) and integration of a PCR product containing the *Candida albicans URA3* gene plus 50 bp of promoter 200 bp to the right of the 3' end of the tRNA gene at *HMRa*. *SET2*, *EAF3*, *RCO1*, *SIR2*, and *DOT1* genes were then replaced in this strain with *MX* markers to generate strains YM2332, YM2333, YM2334, YM2331, and YM2450, respectively. The plasmid BHM225 (*TRP1 CEN*) was also introduced into these strains, as we noted that Trp⁻ strains showed poor growth on 5'-FOA. To find anti-silencing mutations in histones H3 and H4, a *TRP1*-marked plasmid containing *HHT2* and *HHF2* (BHM957) was mutagenized using error-prone PCR on either *HHT2* or *HHF2* and introduced into the strain, where both wild-type and mutant plasmids were maintained using -Lys-Trp media, and mutants conferring extra growth on 5'-FOA were selected. Trp⁺ plasmids were rescued from these mutants and reintroduced into the reporter strain to confirm that growth on 5'-FOA was due to the identified mutation. To quantitate the extent of anti-silencing defects, strains containing both wild-type and mutant plasmids were grown to

saturation in liquid media and then 10-fold serial dilutions were plated onto selective media containing or lacking 5'-FOA. A similar strategy was followed to assess the anti-silencing defects of *set2Δ*, *eaf3Δ*, *rco1Δ*, and *dot1Δ* mutants. To assess whether anti-silencing defects due to histone mutants were Sir dependent, mutant plasmids were introduced into YM2331, which is *sir2Δ*, and plated as above.

Microarray hybridization and analysis: Microarray hybridization was as described (MENEHINI *et al.* 2003), except that mRNA was selected using the QIAGEN (Valencia, CA) Oligotex mRNA mini kit (catalog no. 70022) as per manufacturer's instructions, and data were uploaded onto a NOMAD database (<http://nomad2.ucsf.edu>). Genes with significant expression differences between wild-type and *set2Δ* cells or between wild-type and *set2Δsir4Δ* cells were identified using the significance analysis of microarrays (SAM) package (TUSHER *et al.* 2001) on four replicate experiments with a 10% false discovery rate. The data for transcriptional profiling experiments of *set2Δ* or *set2Δsir4Δ* strains are available at the GEO database (<http://www.ncbi.nlm.nih.gov/geo>) under series accession no. GSE4934.

ChIP: ChIP was performed as described (MENEHINI *et al.* 2003) except that quantitative real-time PCR was performed with SYBR green as a label. Three replicates were performed per experiment using independent cultures for each strain. One microliter of anti-Sir3 antibody was used per sample. Polyclonal anti-Sir3 antibody was generated against the C terminus of Sir3.

RESULTS

Identification of histone residues that are necessary to protect euchromatin from ectopic silencing: Several post-translational modifications have been implicated in anti-silencing. To uncover novel residues in the core histones H3 and H4 that are required for anti-silencing, we devised an unbiased genetic screen. A *C. albicans URA3* gene harboring a truncated promoter was integrated so that its ATG was 250 bp to the right of the tRNA gene right boundary element of *HMRa* (Figure 1a). This reporter construct has been found to be sensitive to mutations that cause the spread of silencing (R. M. RAISNER and H. D. MADHANI, unpublished results). The strain further contained deletions of the two gene pairs encoding for H3 and H4 (*HHT1-HHF1* and *HHT2-HHF2*) complemented by a *LYS2*-marked centromeric plasmid containing the *HHT1-HHF1* locus and an *ADE2* reporter integrated at the chromosome VR telomere (*TELVR*) (PARK *et al.* 2002). We mutagenized the *HHT2* gene by PCR amplification and introduced it into this strain as a *TRP1*-marked centromeric plasmid containing the *HHT2-HHF2* gene pair using a gap repair method. A similar strategy was employed to generate mutations in *HHF2*. Both wild-type and mutant plasmids were maintained using selective media. Dominant mutants with decreased expression of the *URA3* reporter gene were selected on 5'-fluoroorotic acid (5'-FOA), which selects against cells expressing *URA3* (BOEKE *et al.* 1987). We sequenced alleles of *HHT2* and *HHF2* that conferred increased plating efficiency on 5'-FOA and identified eight distinct mutations in H3

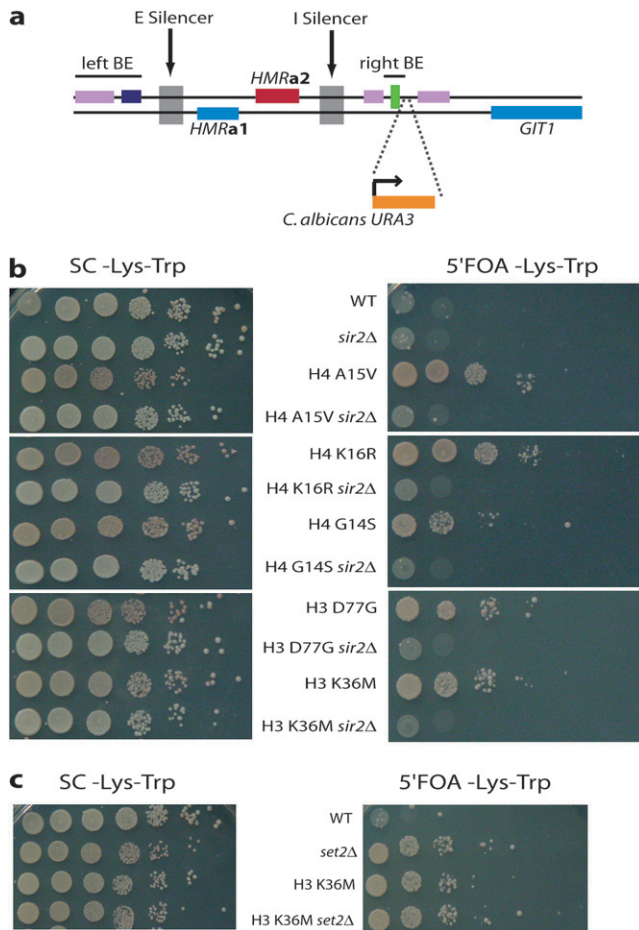


FIGURE 1.—A genetic screen identifies several mutations in H3 and H4 that affect anti-silencing adjacent to *HMRa*. (a) Schematic of the *HMRa* locus and the surrounding region, indicating the site of integration of the *Candida albicans URA3* reporter gene. Ty1 long terminal repeats (LTRs) are shown in light purple, the Ty5 LTR is shown in dark purple, and the tRNA-Thr gene is shown in green. The right boundary element has been defined as the tRNA gene and flanking sequences (DONZE and KAMAKAKA 2001). BE, boundary element. Not to scale. (b) Phenotypes of histone point mutants. To determine the extent of loss of expression from *URA3*, strains were grown to saturation and diluted in 10-fold steps and plated on media containing or lacking 5'-FOA. Examples of increased plating efficiency on 5'-FOA conferred by several mutations in H3 and H4 and suppression of this effect by deletion of *SIR2* are shown. The reporter strain also contains *ADE2* integrated near *TEL1*, which confers a pink or red coloring when silenced and white when expressed. All histone mutants that display increased growth on 5'-FOA also show ectopic telomeric silencing as assayed by darker pink coloration. (c) Comparison of phenotypes of strains with mutations in H3-K36 or *SET2*. Plating of WT and mutant strains on 5'-FOA as described in b is shown.

and six in H4 that cause anti-silencing defects. These alleles are detailed in Table 1, and examples of anti-silencing defects conferred by these mutants are shown in Figure 1b.

To test if these histone mutants caused decreased expression of the *URA3* reporter through Sir-mediated

TABLE 1

Point mutations identified in histones H3 and H4 that confer dominant anti-silencing defects

Histone	Mutation	Sir suppression	Known modification
H3	K36E	Yes	Methylated by Set2
	K36M	Yes	Methylated by Set2
	K36N	Not tested	Methylated by Set2
	Q76R ^a	Not tested	
	D77G ^a	Yes	
	D77V ^a	Yes	
	D77N ^a	Not tested	
H4	D81G ^a	Not tested	
	G14S ^a	Yes	
	A15V ^a	Yes	
	A15T ^a	Yes	
	K16R ^a	Yes	Acetylated by Sas2 and Esa1
	K16M ^a	Yes	Acetylated by Sas2 and Esa1
	G17R Y88C ^a	Yes	

For each mutation, it is noted whether growth on 5'-FOA is suppressed by *sir2Δ* and whether the affected residue is the site of a known post-translational modification.

^a Mutations in or near residues previously identified as necessary for anti-silencing, namely, H4-K16 and H3-K79 (KIMURA *et al.* 2002; VAN LEEUWEN *et al.* 2002; SUKA *et al.* 2002). H3-D77 and -D81 were further previously identified in a screen for mutants that increase telomeric silencing in the absence of Cac1 (SMITH *et al.* 2002).

silencing, we examined the 5'-FOA growth phenotype for several of these mutants in a strain lacking the Sir2 histone deacetylase. Deletion of *SIR2* suppressed the growth phenotype for all histone mutants tested (Figure 1b).

Of the eight residues identified in this screen (Table 1), seven are or are near residues previously shown to be necessary for anti-silencing (indicated by footnote a in Table 1). One identified residue, H3-K36, has not previously been implicated in anti-silencing. We focused on the anti-silencing capacity of H3-K36, as it is the known target of methylation by the histone methyltransferase Set2 (STRAHL *et al.* 2002). The screen identified three different mutations in H3-K36 (Figure 1, b and c, and Table 1). We tested the effect of *SIR2* deletion on the expression of the reporter gene of two K36 mutants, K36M and K36E, and found the phenotype of each to be fully suppressed (Figure 1b and data not shown). Deletion of *SET2* also conferred increased growth on 5'-FOA in the reporter strain, and the double-mutant *set2Δ H3-K36M* strain displayed the same phenotype as the single mutants (Figure 1c), consistent with a model in which methylation of H3-K36 prevents ectopic silencing.

H3-K36 methylation protects euchromatin neighboring telomeres and *HMRa*: To determine whether H3-K36 methylation protects euchromatin from Sir-mediated

silencing in regions other than those flanking *HMRa*, we determined the transcript profiles of WT and *set2Δ* strains using whole-genome DNA microarrays (DERISI *et al.* 1997). Four replicate experiments were performed, and, using the SAM software package (TUSHER *et al.* 2001), genes were identified whose expression changed significantly in the absence of Set2. We identified 290 genes that decreased significantly in expression in the absence of Set2 and 492 genes with significantly increased mRNA levels. We found that regions within 30–40 kb of telomeres were significantly more likely to contain genes with increased expression in *set2Δ* cells than other regions ($P < 0.001$). Additionally, a significant number of genes whose products are involved in oxidoreductase or transporter activity showed increased expression in *set2Δ* cells ($P = 1.87e-14$ and $1.7e-9$, respectively). Regions within 20 kb of telomeres were significantly more likely to contain genes with decreased expression in *set2Δ* cells than genes >20 kb from telomeres (Figure 2a and Table 2). The decreased expression of this telomeric cluster of genes was largely due to ectopic Sir-mediated silencing, since the deletion of *SIR4* suppressed the expression defect caused by *set2Δ* for 26 of 37 of these genes (Figure 2b). In contrast, deletion of *SIR4* suppressed the expression defects of only 37 of 253 genes >20 kb from telomeres that displayed decreased expression and only 8 of the 492 genes that displayed increased expression in *set2Δ* cells. Thus the genes that are dependent on Set2 for their expression can be divided into two classes, those that are silenced by Sir proteins in the absence of Set2, which are enriched near telomeres, and those that display reduced or increased expression in a Sir-independent fashion in the absence of Set2, which are found throughout the genome.

H2A.Z is among other euchromatin-associated factors that regulate the distribution of Sir proteins (MENEHINI *et al.* 2003). In an *htz1Δ* strain, genes near telomeres are enriched for those dependent on H2A.Z for their expression (MENEHINI *et al.* 2003). Since *set2Δ* and *htz1Δ* strains both exhibit this phenotype, we assessed whether they might function in the same regions of the genome to antagonize silencing. We found that while genes that depend on H2A.Z for their expression do not overlap extensively with those that depend on Set2, those within 30 kb of telomeres do overlap significantly between the two data sets (Figure 2c).

The histone methyltransferase Dot1, which catalyzes the methylation of H3-K79 in yeast, has also been implicated in anti-silencing. Deletion of *DOT1* in the anti-silencing reporter strain confers no increased growth on 5'-FOA, and deletion of both *DOT1* and *SET2* restores growth on 5'-FOA to wild-type levels (Figure 3).

Increased Sir protein association in regions flanking silenced chromatin in the absence of K36 methylation: In euchromatin near telomeres and mating-type loci, as in other euchromatic regions, levels of Sir proteins are

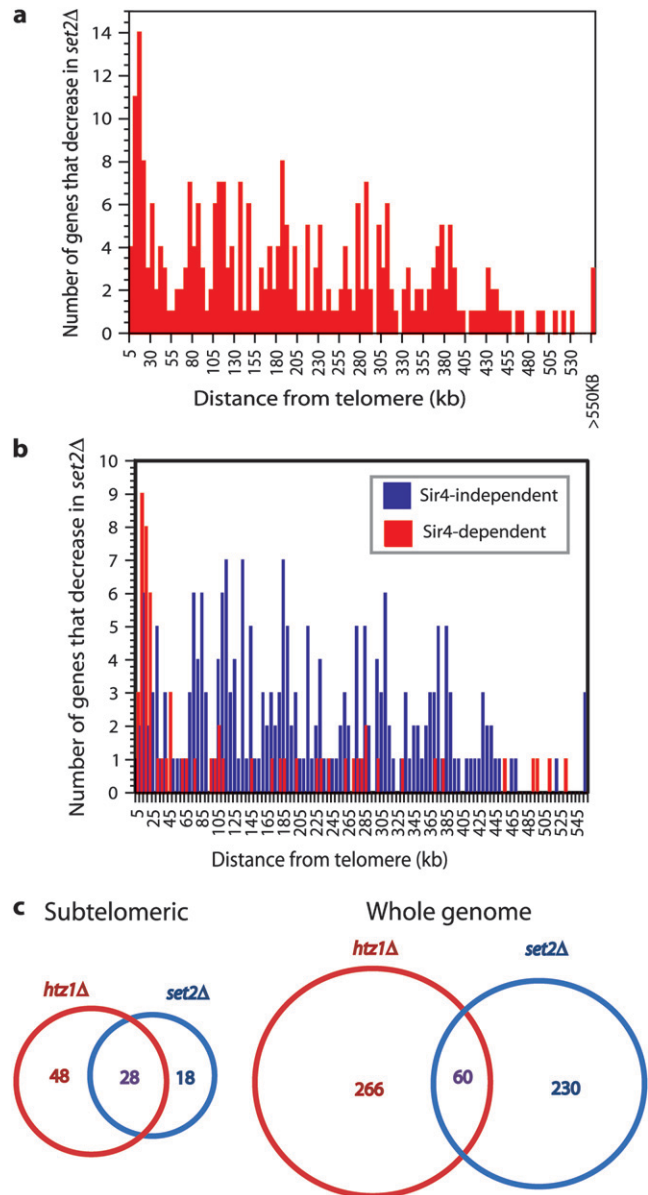


FIGURE 2.—Set2 prevents ectopic silencing of regions bordering heterochromatin genome-wide. (a) Histogram of Set2-dependent genes plotted as a function of distance from telomere. Whole-genome transcriptional profiling of wild-type and *set2Δ* strains was performed as described (MENEHINI *et al.* 2003), using four replicate experiments from separate cultures. Genes with significant changes in expression between wild type and *set2Δ* were determined using SAM (TUSHER *et al.* 2001). (b) Suppression of the subtelomeric gene expression defect by deletion of *SIR4*. Whole-genome transcriptional profiling was performed as in a with WT and *set2Δsir4Δ* strains. Genes from the plot in a were divided into two classes, those that decreased in both *set2Δ* and *set2Δsir4Δ* strains (Sir independent) and those that decreased in a *set2Δ* strain but not in *set2Δsir4Δ* strains (Sir dependent). (c) Venn diagram showing overlap between genes that decrease in *set2Δ* cells and genes that decrease in *htz1Δ* cells (MENEHINI *et al.* 2003). Subtelomeric: genes within 30 kb of telomeres with decreased expression in *htz1Δ* or *set2Δ*. Whole genome: all genes with decreased expression in *htz1Δ* or *set2Δ*. Purple numbers indicate overlap between genes with decreased expression in each strain.

TABLE 2
Genes within 20 kb of telomeres are enriched for those that decrease in expression in *set2Δ* cells

Interval (kb)	Fraction decreased in <i>set2Δ</i>	<i>P</i>
0–10	0.086	0.0099
10–20	0.146	2.419E-09
20–30	0.058	NS
30–40	0.041	NS

P-values for each 10-kb interval were calculated using the χ^2 -test in Microsoft Excel.

normally very low (MENEHINI *et al.* 2003). In mutants such as *htz1Δ*, the spread of Sir proteins beyond its normal boundaries occurs, resulting in ectopic Sir-mediated silencing of genes in these regions (MENEHINI *et al.* 2003). We sought to test if this was the case in a *set2Δ* strain. Two regions were selected for analysis. A group of four genes near *HMRa* showed significant decreases in expression in *set2Δ* strains (indicated by arrows in Figure 4a). Additionally, in the region adjacent to the right telomere of chromosome XIV, 6 of the 7 telomere-proximal genes decreased significantly in *set2Δ* cells (Figure 4a). The expression defect of 5 of 10 genes from these two groups was suppressed by deletion of *SIR4* (indicated gene names in boldface type in Figure 4a). Expression changes of these genes relative to wild type in *set2Δ* or *set2Δsir4Δ* strains are shown in Figure 4b. To determine whether the Sir-dependent repression of these genes in *set2Δ* mutants was caused by direct ectopic binding of the Sir complex, we used ChIP to compare the occupancy of Sir3 in wild-type *vs.* in *set2Δ* strains. We observed an increase over wild-type levels of Sir3 occupancy using PCR probes spanning a 312-bp region starting 302 bp downstream of the 3' end of the tRNA *HMRa* BE (Figure 4c, locus D), consistent with a spread of the Sir complex from *HMRa* beyond normal heterochromatic boundaries. Reproducible increases of Sir3 were found in the region of two other genes that showed decreased expression in the absence of Set2, *ADH7* and *RDS1* (Figure 4c, loci G and I). The telomere-proximal

gene *AIF1* showed a large decrease in expression in *set2Δ* and a corresponding increase in Sir3 levels at the promoter (Figure 4c, locus Z). We also detected increased levels of Sir3 at this locus in an H3 K36A mutant (Figure 4c, right). The relative levels of H3-3meK36 in these regions as assayed by ChIP are shown in Figure 4d (data from POKHOLOK *et al.* 2005). Genes that display decreased expression in a *set2Δ* mutant have high levels of H3-3meK36 with their ORFs, although there is not necessarily a correlation between level of methylation in wild type and degree of silencing in a *set2Δ* mutant. H3-3meK36 also appears to be enriched at the right boundary element of *HMRa*, indicating that it may directly influence boundary element function.

Set2 protects euchromatin from ectopic silencing independently of Rpd3S: H3-K36 methylation is a target for binding by the chromodomain-containing protein Eaf3, which is a component of both Rpd3S and the histone acetyltransferase complex NuA4 (EISEN *et al.* 2001; CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). Recruitment of Rpd3S to chromatin via the interaction between Eaf3 and methylated H3-K36 is necessary to prevent ectopic transcriptional initiation (CARROZZA *et al.* 2005). We therefore assessed whether H3-K36 methylation might also antagonize silencing by recruiting this effector. We tested whether two components of Rpd3S, Eaf3 and Rco1, were required for the anti-silencing function of Set2 by deleting *EAF3* or *RCO1* in the reporter strain used for the histone genetic screen. Deletion of *EAF3* resulted in ~100-fold enhanced plating efficiency over wild type on 5'-FOA media (Figure 5a). This result was confirmed using three independently derived *eaf3Δ* strains (data not shown). In contrast, deletion of *SET2* conferred ~10,000-fold enhanced plating efficiency over wild type on 5'-FOA media. Deletion of *RCO1* resulted in ~≤10-fold enhanced plating efficiency over wild type on 5'-FOA media (Figure 5a). This result was confirmed using two independently derived *rco1Δ* strains (data not shown). Thus, deletion of genes encoding Rpd3S-specific subunits essential for transcriptional fidelity failed to recapitulate the anti-silencing phenotype of mutations in *SET2* or H3-K36.

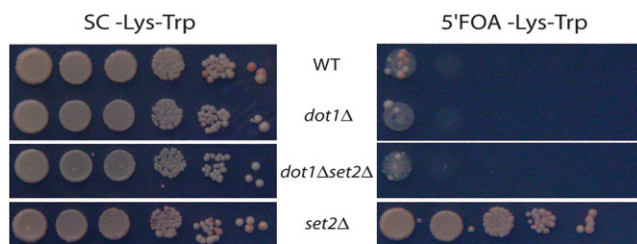


FIGURE 3.—Dot1 does not cause spread of silencing to the right of *HMRa*. Reporter gene assays are shown. *SET2*, *DOT1*, or both were deleted in the reporter strain used for the anti-silencing screen, and strains were plated on media containing or lacking 5'-FOA as described in the Figure 1 legend.

DISCUSSION

Three modified residues and surrounding residues in histones H3 and H4 are important for antagonizing silencing: Through an unbiased genetic screen, we identified residues in the core histones H3 and H4 that are necessary to protect euchromatin from ectopic silencing. Mutations of H4-K16 and surrounding residues were identified, consistent with previous reports that acetylation of K16 by Sas2 prevents spread of Sir proteins at telomeres (KIMURA *et al.* 2002; SUKA *et al.* 2002). We further identified three residues in H3 that cluster near H3-K79 (Table 1), which is methylated by

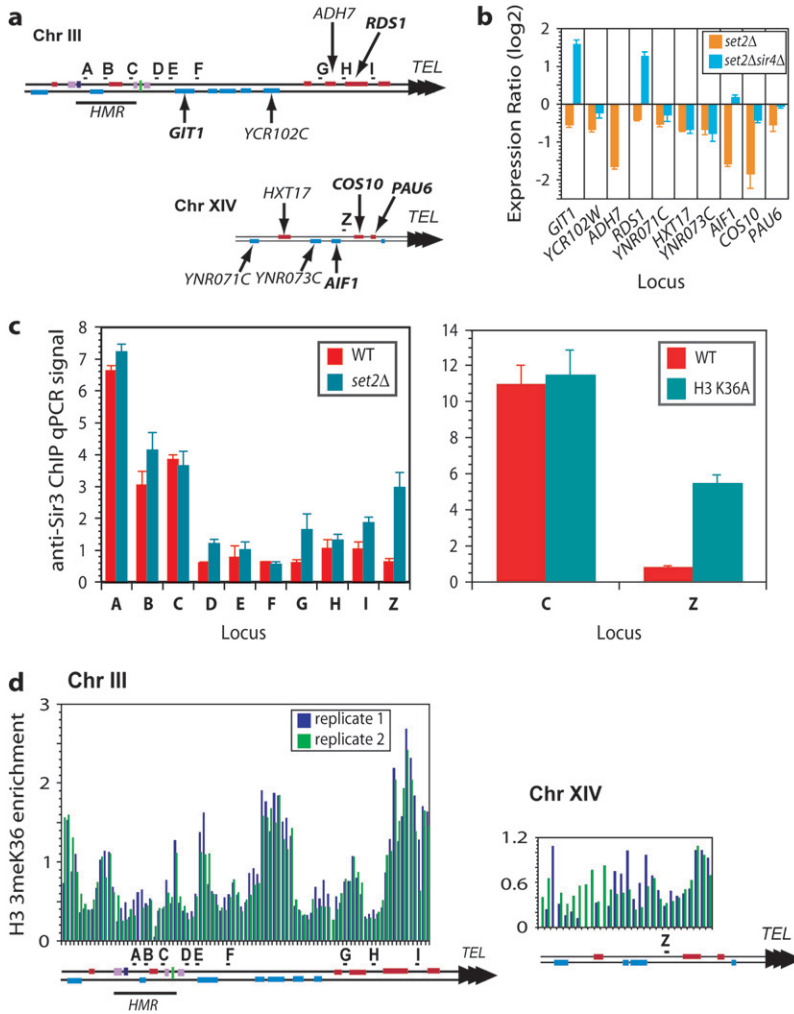


FIGURE 4.—(a) Schematic of the right telomeres and neighboring regions of chromosomes III and XIV. Locations of primer sets used for ChIP in c are denoted by A–I and Z. Arrows indicate genes that decrease significantly in expression in *set2Δ* cells as assayed by expression microarray. Genes that are derepressed in *set2Δsir4Δ* cells are shown in boldface type. This is adapted from Figure 3A in MENEGHINI *et al.* (2003). (b) Effect of deletion of *SET2* or *SET2* and *SIR4* on the expression of *HMR*- and telomere-proximal genes. Shown are the average expression ratios derived from microarray hybridization relative to wild type of the indicated genotypes. Plotted are means and standard error of the mean for four independent experiments performed on each genotype. (c) ChIP using antibodies specific for Sir3. Quantitative PCR was performed using primers as indicated in A. Immunoprecipitation (IP) values were normalized to input values. Plotted are means and standard error of the mean for three independent experiments. (d) Relative enrichments of H3-3meK36 in regions of chromosomes III and XIV studied. Graphs represent data from experiments in POKHOLOK *et al.* (2005), where chromatin IPs against H3 or H3-3meK36 were performed and competitively hybridized to whole-genome microarrays.

Dot1 (FENG *et al.* 2002; LACOSTE *et al.* 2002; VAN LEEUWEN *et al.* 2002). Two of these residues, H3-D77 and -D81, were previously identified in a screen for mutants with increased telomeric silencing (SMITH *et al.* 2002). Consistent with these results, loss of methylation at H3-K79 causes increase of Sir2 and Sir3 occupancy at Y' subtelomeric elements (VAN LEEUWEN *et al.* 2002). A previous screen for histone mutations identified several residues surrounding H3-K79, including H3-K79 itself, as important for silencing (PARK *et al.* 2002). This study identifies a nonoverlapping set of residues near H3-K79 that are important for anti-silencing. Removal of Dot1, the H3-K79 methyltransferase, does not confer increased growth on 5'-FOA in our anti-silencing reporter strain. Further, deletion of *DOT1* suppresses the anti-silencing phenotype of *set2Δ* in the reporter strain (Figure 3). This may be due to negative regulation of Dot1 by Set2, although it is more likely due to a general redistribution of Sir proteins throughout the genome in the absence of H3-K79 methylation, as has been observed by microscopy in spread mitotic and pachytene nuclei (SAN-SEGUNDO and ROEDER 2000).

A novel function for Set2-mediated methylation of H3-K36: We identified H3-K36 as necessary for anti-silencing via the reporter-based genetic screen. This residue had not previously been implicated in anti-silencing. We further demonstrated that methylation of this residue by Set2 is necessary to protect euchromatin from ectopic silencing by spread of Sir proteins from heterochromatin. Set2 is recruited to Pol II-transcribed genes via association with the elongating form of Pol II (LI *et al.* 2002, 2003; KROGAN *et al.* 2003; SCHAFT *et al.* 2003; XIAO *et al.* 2003). Set2 was recently shown to inhibit transcriptional initiation within coding sequences by recruiting Rpd3S and promoting deacetylation of histone tails (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). In this work, we identify a distinct function for K36 methylation. We found that both mutation of *SET2* and several different amino acid replacements of K36 cause Sir-mediated transcriptional silencing of an *HMR*-adjacent reporter gene. Further, we used genomewide analysis to show that Set2 protects subtelomeric euchromatin from Sir-dependent silencing. In the absence of Set2, genes within 20 kb of telomeres

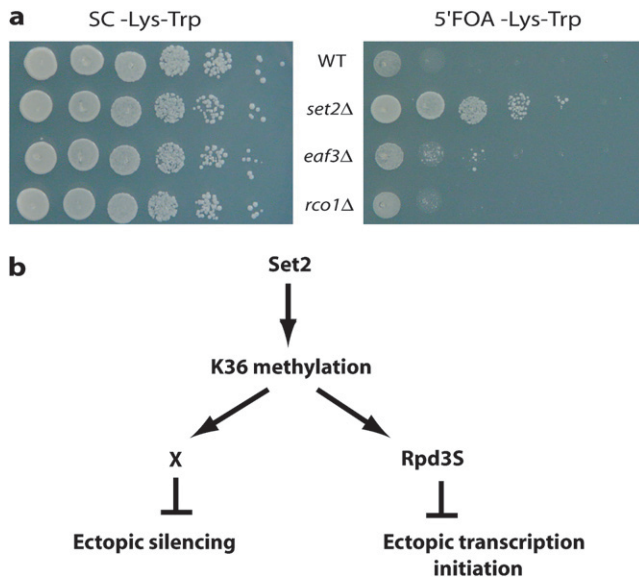


FIGURE 5.—Set2 antagonizes spread of silencing independently of Rpd3S. (a) Reporter gene assays. *SET2*, *RCO1*, or *EAF3* were deleted in the reporter strain and plated as in Figure 1. (b) A genetic model that proposes distinct pathways that mediate the roles of H3-K36 methylation in anti-silencing and repression of ectopic initiation sites.

decrease significantly in expression. Concomitantly, Sir3 levels are increased in euchromatic regions in the absence of K36 methylation. Thus we conclude that methylation of H3-K36 antagonizes silencing near telomeres and silent mating-type loci by restricting spread of Sir proteins and further promotes expression of some telomere-distal genes independently of the Sir complex.

Not all genes with decreased expression in a *set2Δ* strain show increased Sir3 occupancy as assayed by ChIP. For example, *GIT1* shows decreased expression in the absence of Set2 by microarray that is dependent on Sir4 (Figure 4, a and b) but no detectable increase in Sir3 in its promoter (Figure 4c, locus F). This discrepancy may indicate that Sir proteins can silence at a distance, or it may simply reflect a difference in detection thresholds between the ChIP and transcriptional profiling technologies. Similarly, the increase in Sir3 occupancy at euchromatic sites adjacent to silenced loci (Figure 4c) leads us to posit a model in which the Sir complex spreads from heterochromatin into adjacent euchromatin in the absence of H3-K36 methylation. However, we can detect more Sir3 at locus G than at locus H (Figure 4c), despite the fact that locus H is more telomere proximal than locus G. This leads us to believe that the spread of silencing proteins does not necessarily proceed in a linear gradient. Alternatively, this discrepancy could be due to noise in the ChIP assay.

H2A.Z was previously characterized in our laboratory as a general anti-silencing factor (MENEGHINI *et al.* 2003), similar to the role that we describe here for Set2. We found that a significant fraction of genes

within 30 kb of telomeres with decreased expression in the absence of Set2 also show decreased expression in the absence of H2A.Z (28 of 46). However, of all the genes with decreased expression in a *set2Δ* strain (290), only 60 also show decreased expression in an *htz1Δ* strain. Consistent with this lack of overlap, *htz1Δ* and *set2Δ* strains display synthetic lethality (KROGAN *et al.* 2003), and H2A.Z and methylated H3-K36 are present in distinct regions of genes (KROGAN *et al.* 2003; GUILLEMETTE *et al.* 2005; RAISNER *et al.* 2005; RAO *et al.* 2005; POKHOLOK *et al.* 2005; ZHANG *et al.* 2005). We conclude that H2A.Z and Set2 antagonize silencing in the same regions of the genome. Their synthetic lethality may reflect independent anti-silencing mechanisms, although it is possible that H2A.Z and Set2 are present in two pathways that converge on a common downstream factor or that the synthetic lethality represents redundancy of another function unrelated to anti-silencing.

Histone methylation on K36 has both Rpd3S-dependent and independent functions: The only known effector for K36 methylation is the Rpd3S complex, which associates with methylated H3-K36 in a manner that requires the chromodomain of the Eaf3 subunit (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). It has been proposed that the Eaf3 chromodomain specifically recognizes the modified residue *in vivo* (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). We observed that the *eaf3Δ* mutation conferred considerably weaker growth on 5'-FOA of the reporter strain than the *set2Δ* mutation and that the *rco1Δ* strain was almost indistinguishable from wild type on 5'-FOA. Therefore, Set2-mediated anti-silencing acts at least in part in a pathway that does not require the recruitment of Rpd3S (Figure 5b). It is possible that the *eaf3Δ* mutation confers an anti-silencing defect due to the association of Eaf3 with the NuA4 complex, which also antagonizes silencing (OKI *et al.* 2004), and not Eaf3's association with Rpd3S, since absence of the Rpd3S-specific subunit Rco1 conferred no detectable anti-silencing defect. Taken together, our data imply that methylation of H3-K36 functions through at least two different effector mechanisms.

Links between H3-K36 methylation and cancer: There are three known homologs of *SET2* found in humans, *NSD1*, *NSD2*, and *NSD3* (SCHNEIDER *et al.* 2002; RAYASAM *et al.* 2003). All three genes have been implicated in cancer. Rearrangements of the genetic sequences of *NSD1* and *NSD3* that result in truncations are associated with acute myeloid leukemia, and a fusion of *NSD2* that is thought to cause overexpression of the gene is associated with multiple myeloma (SCHNEIDER *et al.* 2002). *NSD3* is also rearranged in several cancerous cell lines (SCHNEIDER *et al.* 2002). It is possible that ectopic gene silencing occurs in response to defects in K36 methylation in humans as well, which could contribute to cancer initiation and progression.

The authors thank Jef Boeke for strains and plasmids. We are especially grateful to Ryan Raisner for construction of the anti-silencing reporter and assistance with anti-silencing assays. We thank Nguyen Nguyen for technical assistance, Shivkumar Venkatasubrahmanyam for advice on microarray experiments and analysis, and Marc Meneghini for assistance with ChIP experiments. We are grateful to Barbara Panning, Marc Meneghini, and Sigurd Braun for critical reading of this manuscript and to all members of the Madhani lab for valuable discussions and suggestions. This work was supported by a National Science Foundation Graduate Research Fellowship to R.T., a Lymphoma and Leukemia Society Scholar Award to H.D.M., and a grant from the National Institutes of Health to H.D.M. (GM071801).

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Communicating editor: J. TAMKUN