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Dot1p Modulates Silencing in Yeast by Methylation of the Nucleosome Core

Fred van Leeuwen,[1] Philip R. Gafken,[2] and Daniel E. Gottschling[1,3]
[1]Division of Basic Sciences
[2]Proteomics Facility
Fred Hutchinson Cancer Research Center
1100 Fairview Avenue North
Seattle, Washington 98109

Summary

***DOT1* was originally identified as a gene affecting telomeric silencing in *S. cerevisiae*. We now find that Dot1p methylates histone H3 on lysine 79, which maps to the top and bottom of the nucleosome core. Methylation occurs only when histone H3 is assembled in chromatin. In vivo, Dot1p is solely responsible for this methylation and methylates ~90% of histone H3. In *dot1*Δ cells, silencing is compromised and silencing proteins become redistributed at the expense of normally silenced loci. We suggest that methylation of histone H3 lysine 79 limits silencing to discrete loci by preventing the binding of Sir proteins elsewhere along the genome. Because Dot1p and histone H3 are conserved, similar mechanisms are likely at work in other eukaryotes.**

Introduction

The eukaryotic genome is organized into active domains and highly compacted, heterochromatic domains. One property of heterochromatin is that genes located within it, or close to it, can become silenced in a sequence-independent manner (reviewed in Moazed, 2001). Once established, the silenced state can be heritably passed on to progeny cells for many cell generations. Several components of heterochromatic and silenced domains have been identified in a number of organisms. While there are notable differences in the components between species, the underlying mechanisms of silencing appear to be similar (Moazed, 2001).

The foundation of chromatin is the nucleosome, composed of ~147 base pairs of DNA wrapped around two pairs of four histone proteins H2A, H2B, H3, and H4 (reviewed in Kornberg and Lorch, 1999). The histones contain a highly structured histone-fold domain that contributes to the nucleosome core. They also have less structured NH_2- and COOH-terminal tails that extend beyond the nucleosome core (Luger et al., 1997) and are readily available for interaction with other proteins. These tails are sites of multiple posttranslational modifications that can affect chromatin structure and function (reviewed in Jenuwein and Allis, 2001).

The formation of silent chromatin is driven by cooperative interactions between DNA sequences required for silencing (silencers), the proteins that bind to silencers, and silencing proteins that interact with the DNA binding proteins and with the nucleosomes (reviewed in Gasser and Cockell, 2001; Moazed, 2001). In this network of interactions, silencing is affected by posttranslational modifications of the histone tails, such as acetylation and methylation (reviewed in Jenuwein and Allis, 2001; Zhang and Reinberg, 2001). Within active chromatin, lysine (Lys) residues in the tails of histone H3 and H4 are usually hyperacetylated, but in silent chromatin, the tails are hypoacetylated. Methylation of the tails of histone H3 and H4 occurs at Lys or arginine (Arg) residues. While Arg methylation is associated with transcriptional activation, the function of Lys methylation seems to be dependent on the specific residue in the histone tail (reviewed in Jenuwein and Allis, 2001; Zhang and Reinberg, 2001).

In the budding yeast *Saccharomyces cerevisiae,* gene silencing occurs at the silent mating-type loci *HML*α and *HMR*a (*HM*), at telomeres, and at the ribosomal RNA gene array (rDNA). Silencing at the *HM* loci and telomeres requires a complex of the silencing proteins Sir2p, Sir3p, and Sir4p (Sir complex or SIR), whereas only Sir2p is involved at the rDNA (reviewed in Gasser and Cockell, 2001). Sir2p is an NAD-dependent histone deacetylase, which is required to achieve the hypoacetylated state of the histone H3 and H4 tails within silent chromatin (reviewed in Gottschling, 2000). The Sir complex is recruited to the *HM* loci by the DNA binding proteins Rap1p, Abf1p, and the origin of replication complex (ORC; reviewed in Gasser and Cockell, 2001). At telomeres, where switching between active and silent states can occur, the Sir complex is recruited by the telomeric DNA binding proteins Rap1p and yKU (reviewed in Gasser and Cockell, 2001; Moazed, 2001). The Sir complex spreads from the telomere inward along the chromosome, but the manner and distance, in which it spreads are influenced by the DNA sequences adjacent to the telomeres (Fourel et al., 1999; Pryde and Louis, 1999; Renauld et al., 1993). Two families of subtelomeric elements, X and Y′, are located immediately adjacent to telomeres and have DNA elements within them that are reported to either block or enhance the propagation of silencing (Fourel et al., 1999; Pryde and Louis, 1999). Within the rDNA, Sir2p together with Cdc14p is targeted to the nucleolus by Net1p (reviewed in Gasser and Cockell, 2001). Sir3p and Sir4p are not normally located at the rDNA.

In a search for novel regulators of gene silencing, we previously isolated *DOT1* as a high-copy disruptor of silencing at telomeres, *HM* loci, and rDNA (Singer et al., 1998). In vegetative cells, Dot1p (also known as Pch1p) is localized in the nucleus and associates with chromatin in chromosome spreads (San-Segundo and Roeder, 2000). Loss of *DOT1* function (*dot1*Δ) has several effects on silencing, including a significant reduction in silencing of a telomeric marker gene (Singer et al., 1998), a more modest reduction in *HM* loci silencing (Singer et al., 1998), and a change in the distribution of Sir3 protein staining on chromosomes (San-Segundo and Roeder, 2000). Recently, by transcript array analysis of a *dot1*Δ strain, two trends were observed (Hughes et al., 2000):

[3]Correspondence: dgottsch@fhcrc.org

telomere-proximal genes such as the *COS* genes were expressed at higher levels, consistent with the effects seen on marker genes inserted near telomeres (Singer et al., 1998), and there was a modest reduction in expression of RNAs encoded within the telomere-associated Y′ elements. Here, we report our biochemical analysis of Dot1p, which reveals that modification of the nucleosome core impacts the organization of the genome into active and silent chromatin domains.

Results

Mutation of the Putative Methyltransferase Domain of Dot1p Inactivates Its Silencing Function

The C-terminal half of Dot1p is homologous to putative proteins identified in other organisms (Figure 1A). We found that this conserved domain has features of a binding site for S-adenosyl-methionine (AdoMet), the methyl donor for many methylation reactions (Kagan and Clarke, 1994). It has recently been suggested that the predicted secondary structure of Dot1p has a methyltransferase (MT) fold similar to that of the PRMT family of Arg MTs (Dlakic, 2001). These observations raised the possibility that Dot1p might be an AdoMet-dependent MT.

In the PRMT family of MTs, the key residue in binding of AdoMet is a highly conserved glycine (Gly) in motif I (Zhang and Reinberg, 2001). To test if the putative MT fold is important for the silencing function of Dot1p, its corresponding Gly (see arrow in Figure 1A) was changed to Arg (G401R) or alanine (Ala; G401A). Single-copy plasmids that contained either of these mutant alleles or the wild-type (wt) *DOT1* gene were transformed into a *DOT1* deletion strain (*dot1*Δ), which contained a telomeric URA3 gene that served as a reporter for telomeric silencing (Figure 1B; Gottschling et al., 1990). The plasmid containing the wild-type *DOT1* gene complemented the deletion of *DOT1* (Figure 1C). In contrast, the plasmids containing the *dot1-G401R* or *G401A* allele showed little or no complementation, even though their gene products were expressed at the same level as the wild-type protein (Figure 1C). This suggested that the putative MT domain of Dot1p is required for wild-type silencing at telomeres (Figure 1C).

We next tested whether the AdoMet binding site was required for the overexpression phenotype of *DOT1*. High-copy plasmids containing *DOT1* under control of the *GAL1* promoter were transformed into a wild-type strain with a telomeric *URA3* gene. While cells overexpressing wild-type *DOT1* showed nearly complete loss of telomeric silencing (Figure 1D), overexpression of *dot1-G401A* or *dot1-G401R* had only a very modest reduction in silencing. These data suggest that *DOT1* has a MT activity that is required for its high-copy disruption of silencing. Furthermore, overexpression of the Dot1 mutant proteins did not have a strong dominant-negative effect on silencing, suggesting that the mutants did not effectively compete with the endogenous wild-type protein for binding to putative Dot1p-interacting factors.

Dot1p Is a Methyltransferase

To directly test if Dot1p has intrinsic MT activity, recombinant Dot1 protein (rDot1p) was incubated with a variety of prospective substrates and ^{3}H-AdoMet. While a previously characterized Arg MT, rHmt1p (McBride et al., 2000), methylated several purified proteins, including calf-thymus histones, rDot1p did not methylate any of the purified substrates tested (data not shown and see below). However, when rDot1p was applied to a whole cell or nuclear extract from a yeast strain lacking the endogenous *DOT1* gene (to avoid modification of substrate by endogenous Dot1p), a single protein of ~15 kDa was labeled by rDot1p (Figure 2A, data not shown). In contrast, equal amounts of the mutant protein rDot1p-G401R had no detectable MT activity (Figure 2A). Upon longer exposure, additional methylated proteins were detected using extracts from *dot1*Δ or wild-type cells as a substrate, but none of those were specific for Dot1p (data not shown). Thus, Dot1p is a protein MT, and mutation of the highly conserved Gly401 abolishes its MT activity and its silencing function.

Dot1p Methylates Lysine 79 of Histone H3

The apparent molecular weight of the rDot1p substrate and its presence in the nuclear extract suggested that it might be a histone. Therefore, after combining a yeast nuclear extract with rDot1p and ^{3}H-AdoMet, the histone proteins were purified and subsequently separated by reverse-phase HPLC (RP-HPLC). The entire ^{3}H label was recovered in the histone preparation and coeluted with the fractions containing histone H3 (Figure 2B).

In order to determine which residue was methylated, HPLC-purified radiolabeled histone H3 was mixed with excess purified histone H3 from wild-type cells and treated with trypsin. When tryptic fragments were separated by RP-HPLC, the entire radioactivity was recovered in one fraction containing one abundant absorbance peak (Figure 2C). This fraction was analyzed by nano-liquid chromatography electrospray ionization mass spectrometry (LC-ESMS), and four doubly charged ion species were detected in a single chromatographic peak (Figure 2D). The smallest ion species, with a mass-to-charge ratio (*m/z*) of 668.5, corresponded to the histone H3 peptide 73-EIAQDFKTDLR-83. The other peaks increased by increments of 7 *m/z* units, which corresponds to the *m/z* of a methyl group in doubly charged ions (*14/2*). Thus, the four peaks appeared to represent the histone H3 peptide in unmodified, mono-, di-, and trimethylated forms. Sequencing of each of the four peptides by tandem mass spectrometry (MS/MS) confirmed their identity and mapped the modification in each peptide to Lys79 of histone H3 (Figures 2E and 2F). Since acetylation and trimethylation of Lys result in an identical nominal mass increase (+21 *m/z* in doubly charged ions), it was possible that the *m/z* 689.6 ion represented, at least in part, acetylation of Lys79. However, further analysis indicated that Lys79 was trimethylated and not acetylated (see below).

The NH_2-terminal tail of histone H3 is the site of numerous posttranslational modifications and is critical in the formation of silent chromatin (reviewed in Jenuwein and Allis, 2001). Furthermore, there is ample evidence that some of these modifications may regulate subsequent modifications on the same histone. Therefore, we tested whether the tail was important for methylation of histone H3-Lys79 by incubating rDot1p with a nuclear extract

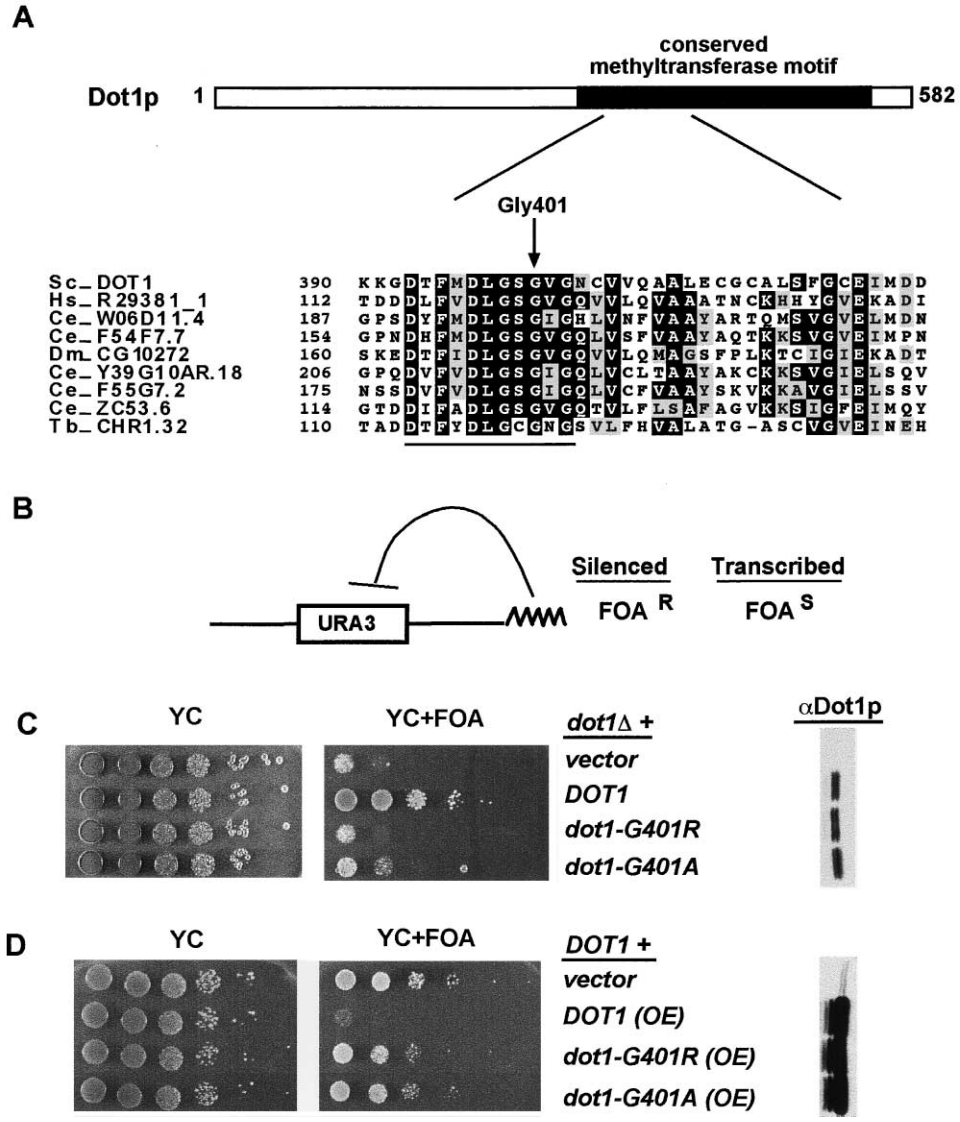

Figure 1. The Putative Methyltransferase Motif Is Essential for the Silencing Function of *DOT1*

(A) The conserved COOH-terminal half of Dot1p contains an AdoMet-dependent MT motif (black box). Alignment and boxshading were performed with ClustalW 1.74 and Boxshade 3.2, respectively, at http://www.ch.embnet.org. Black boxes indicate identity, gray boxes indicate similarity if there is ≥50% consensus. The alignment includes putative ORFs from humans (Hs), worms (Ce), flies (Dm), and trypanosomes (Tb). Numbers at the left of each sequence indicate position of the first residue used in the alignment. The arrow indicates the highly conserved Gly401 in motif I (underlined).

(B) Telomeric silencing of a *URA3* reporter gene was measured by growth of colonies on media containing 5-fluoroorotic acid (FOA). When *URA3* is silenced, cells are resistant to FOA. When *URA3* is expressed, cells convert FOA into a toxic product and are sensitive to FOA (van Leeuwen and Gottschling, 2002).

(C) A single-copy plasmid with no insert (vector), wild-type *DOT1*, *dot1-G401R*, or *dot1-G401A* was transformed into *dot1*Δ strain UCC7121. To measure telomeric silencing, cells were plated in a 10-fold dilution series on selective media (YC) in the absence or presence of FOA. Dot1p protein levels were examined by Western blot analysis.

(D) To test the effect of *DOT1* mutations on high-copy disruption of silencing, *DOT1* was overexpressed (OE) in wild-type strain UCC1091 by the galactose-inducible *GAL1* promoter. Cells were plated as in (C) on media containing galactose.

from a strain containing a mutant histone H3 gene that lacked the NH_2-terminal tail (H3Δ2-30). Histone H3 without amino acids 2–30 was methylated to the same level as wild-type histone H3 (Figure 2G). Thus, the tail of histone H3 was not necessary for Dot1p methylation of Lys79.

Next, Lys79 was changed to Ala (K79A) in order to independently evaluate whether this residue was the site of Dot1p methylation and to determine if it was the only site methylated. We used the faster migrating histone H3Δ2-30 mutant as an internal control for our analysis of the K79A mutant. When nuclear extract containing histone H3-K79A mutant protein was mixed with an equal amount of nuclear extract of the histone H3Δ2-30 tail mutant, only the tailless mutant was labeled (Figure 2G). Thus, K79A did not serve as a substrate for

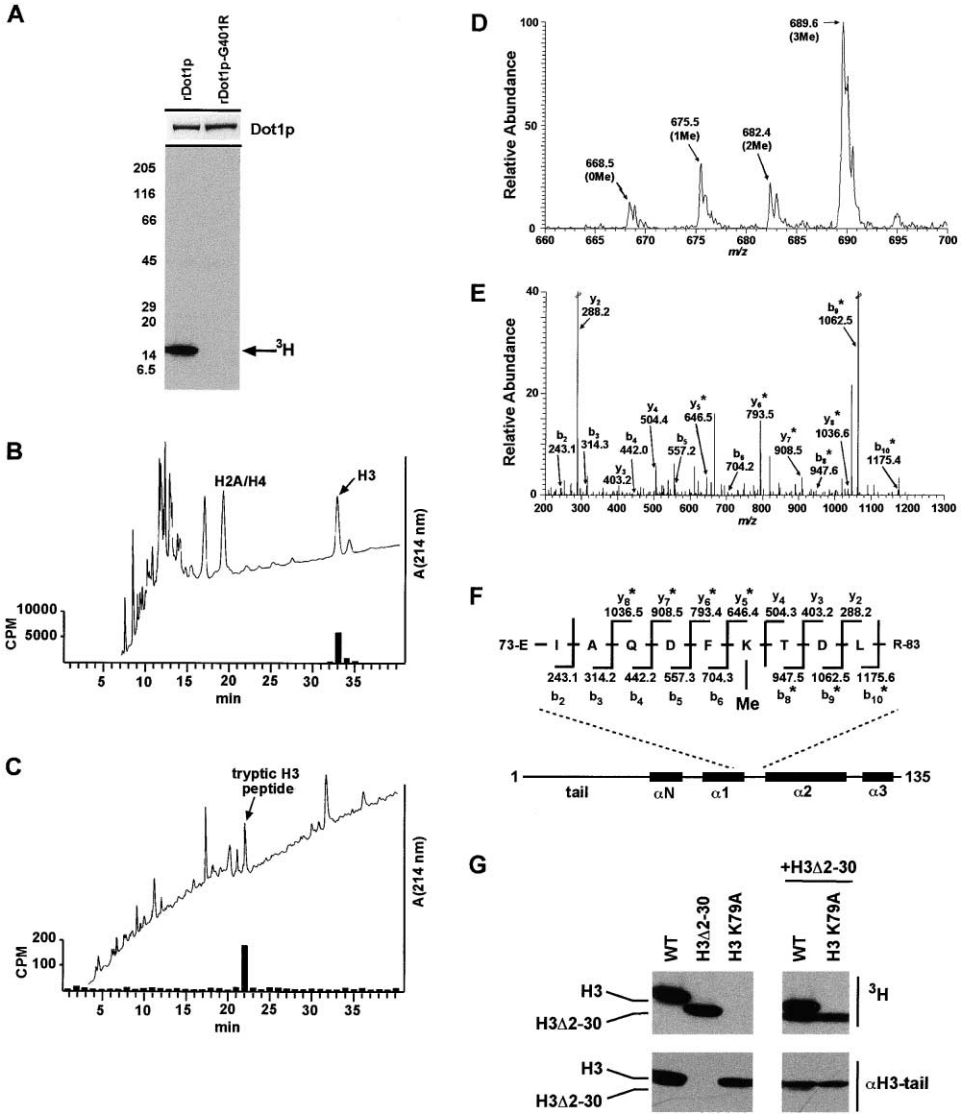

Figure 2. Dot1p Methylates Histone H3 on Lysine 79 In Vitro

(A) Recombinant Dot1p (rDot1p) or mutant rDot1p-G401R was incubated with nuclear extract from strain UCC7121 (*dot1*Δ) and ^{3}H-AdoMet. rDot1 protein levels were examined by Coomassie staining (top of lanes-Dot1p). Radiolabeled (methylated) proteins were separated by SDS-PAGE, transferred to membrane, and detected by autoradiography (^{3}H).

(B) Histones purified from a large-scale MT reaction were separated by RP-HPLC. Elution of ^{3}H-methylated proteins and peptides was monitored by absorbance at 214 nm [A(214 nm)] and scintillation counting [CPM, black bars, plotted on the same time scale as A(214 nm)].

(C) The fractions containing ^{3}H-methylated histone H3 were mixed with excess purified histone H3 from wild-type strain UCC1091 to facilitate detection, lyophilized, and then digested with trypsin. The tryptic peptides were separated by RP-HPLC and identified as in (B).

(D) Fraction 22 in (C) was lyophilized and analyzed by LC-ESMS. A mass chromatogram of the digestion mixture was collected and four ion species of *m/z* 668.5, 675.5, 682.4, and 689.6 were measured in a single chromatographic peak. Differences between these peaks of 7 *m/z* indicated that the ion species represented doubly charged unmodified, mono-, di-, and trimethylated forms (0Me, 1Me, 2Me, and 3Me), respectively, of the histone H3 peptide 73-EIAQDFKTDLR-83.

(E) MS/MS was performed on ion species *m/z* 668.5, 675.5, 682.4, and 689.6 to verify their identity with data for *m/z* 675.5 shown. The resulting spectrum shows singly charged b-type and y-type ion species that were used for identification. Fragment ions labeled with asterisks indicate mass fragments that are modified by a single methyl group.

(F) MS/MS data was compared to the expected fragmentation pattern for 73-EIAQDFKTDLR-83. The expected fragment masses are labeled for those b- and y-type ions observed in (E) that verify the identity of the peptide. The mass difference between y_5* and y_4 locates the site of methylation to Lys79, which is located in the core of histone H3, in loop 1 between α-helix 1 and α-helix 2 (Luger et al., 1997; White et al., 2001).

(G) rDot1p MT activity was tested on histone H3 in nuclear extracts of *dot1*Δ strains containing wt histone H3 (UCC7121), H3Δ2-30 (UCC7220), or H3K79A (UCC7224). Methylated histone H3 was detected by autoradiography (^{3}H). Histone H3 protein levels were determined by incubation of the same membranes with antibodies against the tail of histone H3, followed by detection by chemiluminescence (α-H3-tail). The epitopes for these antibodies are missing from the histone H3Δ2-30 protein.

rDot1p. These results indicate that rDot1p is a histone methyltransferase (HMT), which methylates histone H3 exclusively at Lys79 in vitro.

Dot1p Is the Sole In Vivo Methyltransferase for Lys79 on Histone H3

Given Dot1p's proficient methylation of histone H3-Lys79 in vitro, we next determined whether Dot1p methylates this site in vivo, and if so, to what level. Since Dot1p is important for silencing and silencing in yeast only occurs in a small fraction of the genome, we anticipated the methylation of Lys79 to be in low abundance. Histone H3 was purified from a wild-type and a *dot1*Δ strain, and the methylation status of Lys79 was analyzed as before with one modification. The isolated histone H3 proteins were digested with endoproteinase Arg-C instead of trypsin to avoid cleavage at Lys79 and thus ensure equal recovery of unmodified and methylated forms of the 73-EIAQDFKTDLR-83 peptide. As opposed to our expectation, methylation in wild-type cells was very abundant; approximately 90% of all Lys79 was methylated (Figure 3). Methylation of Lys79 occurred in three different forms: mono-, di-, and trimethylation, with the latter being the most abundant (Figure 3). In *dot1*Δ cells, no methylation of histone H3-Lys79 was detected (Figure 3). Only the unmodified peptide was recovered, indicating that no other HMT in yeast methylated Lys79 to a detectable level.

To confirm that Dot1p was directly involved in mono-, di-, and trimethylation of Lys79, unmethylated histone H3 in nuclear extracts from a *dot1*Δ strain was methylated in vitro by rDot1p in the presence of excess (nonradioactive) AdoMet. This in vitro methylation of histone H3 restored the methylation to a pattern very similar to that of histone H3 isolated from wild-type *DOT1* cells (Figure 3), supporting the idea that Dot1p was directly responsible for mono-, di-, and trimethylation of histone H3-Lys79 in vitro and in vivo. Furthermore, we conclude that the *m/z* 689.6 ion (Figure 2D) represented only the trimethylated and not an acetylated peptide, because no methylation or acetylation of Lys79 was found in *dot1*Δ cells and rDot1p could trimethylate Lys79 in vitro.

Overexpression of Dot1p in vivo, which disrupted silencing in an HMT-dependent manner (Figure 1D), led to an overall increase in the level of methylation (to ~98%), with a striking predominance of the trimethylated form (~90%; Figure 3, wt + *DOT1* [OE]). The strain carrying the vector alone (Figure 3, wt + vector), grown under the same conditions, had a methylation pattern very similar to that of wild-type cells without a plasmid.

Together, the results show that Dot1p is the sole histone H3-Lys79 HMT in yeast and that the expression level of Dot1p determines the amount of Lys79 methylation within cells.

Dot1p Methylates Histone H3 in Chromatin

All the in vitro methylation by rDot1p described above was done on histone H3 present in chromatin in yeast nuclear extracts. As noted earlier, purified histones from calf thymus did not serve as a substrate (Figure 4). Therefore, histone H3 of a *dot1*Δ strain was purified from the yeast nuclear extract (as described in Figure 2) and assayed as a substrate for methylation by rDot1p.

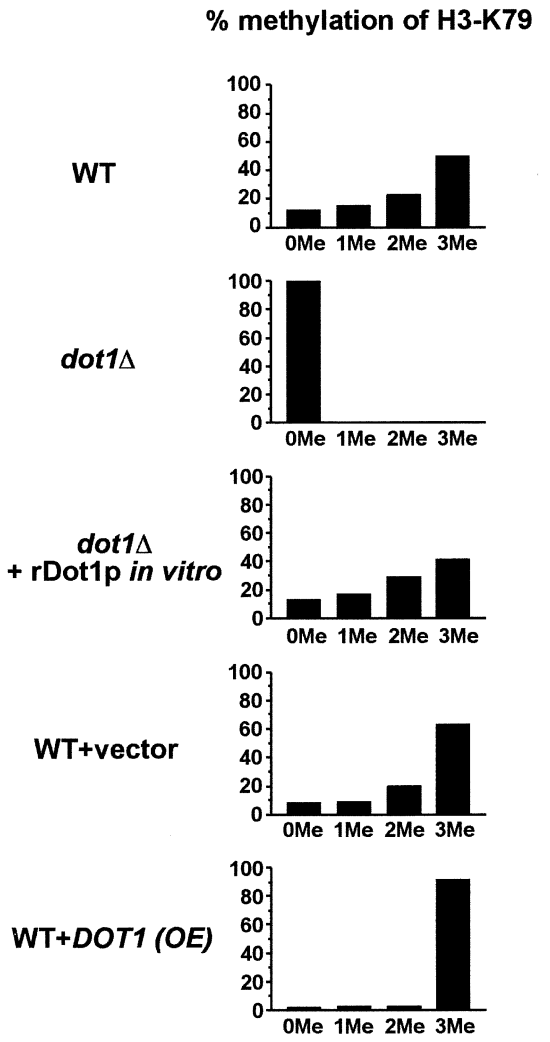

Figure 3. Dot1p Modifies the Majority of Histone H3-Lys79 in Yeast by Mono-, Di-, or Trimethylation

The relative levels of unmodified, mono-, di-, and trimethylated histone H3-Lys79 in vivo (0Me, 1Me, 2Me, and 3Me, respectively) were measured in wt (UCC1091), *dot1*Δ (UCC7121), *dot1*Δ + rDot1p in vitro (histone H3 from UCC7121, methylated in a nuclear extract in vitro by rDot1p). WT+vector and WT+DOT1 (OE) were grown in media containing galactose to test overexpression of *DOT1* in vivo (see Figure 1D). Endo Arg-C digestion mixtures of RP-HPLC-purified histone H3 were analyzed by MS as shown in Figure 2.

Whereas the same pool of histone H3 was efficiently methylated in the nuclear extract, no methylation of the purified yeast histone H3 was detected (Figure 4), just as it was for the histone H3 from calf thymus. Even when the purified yeast histone H3 was mixed with nuclear extracts of a histone H3Δ2-30 mutant strain, it was not methylated by rDot1p (Figure 4). This latter experiment suggested that there was not a diffusible cofactor in the nuclear extract that mediated methylation by rDot1p.

To determine if methylation by Dot1p required a particular chromatin structure, chromatin in yeast nuclei was converted into mononucleosomes by extensive treatment with micrococcal nuclease, which preferentially cleaves DNA in chromatin in the linker region be-

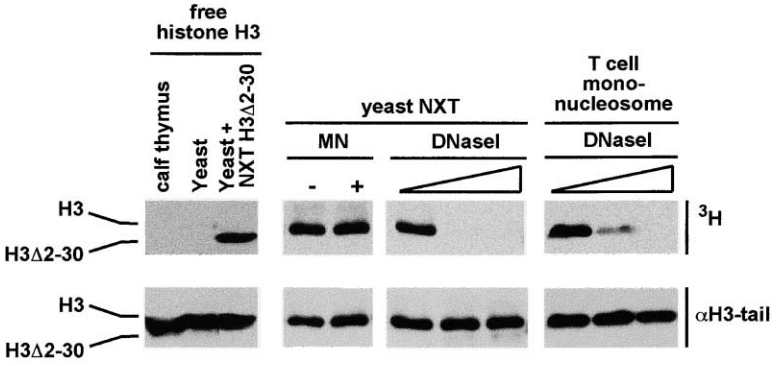

Figure 4. Dot1p Methylates Histone H3 in Chromatin

rDot1p was incubated with equal amounts of histone H3 purified from calf thymus, or from a yeast *dot1*Δ strain (UCC7121) in the absence or presence of nuclear extract from strain UCC7220 (*dot1*Δ, H3Δ2-30). Nuclear extract from the UCC7121 was pretreated with 0 or 0.1 U/μl micrococcal nuclease (MN, Roche) for 5 min or with 0, 0.03, or 0.3 U/μl DNase I (Worthington) for 10 min at 37°C. Mononucleosomes were isolated from mouse T cells and pretreated with 0, 0.03, or 0.3 U/μl DNase I for 10 min at 37°C. Methylated histone H3 was detected by autoradiography (^{3}H). Histone H3 protein levels were determined as in Figure 3 (α-H3-tail).

tween nucleosomes. The histone H3 in these mononucleosomes was as good a substrate for methylation as the nucleosome arrays present in the nuclear extract (Figure 4), thus eliminating the possibility that higher-order chromatin structure was needed for Dot1p activity. In stark contrast, degradation of DNA in chromatin by treatment with DNase I, which destroys the nucleosome, led to a loss of methylation of histone H3 by rDot1p even though levels of histone H3 in the extract did not change (Figure 4). As independent verification that Dot1p only worked on nucleosomes, mononucleosomes were purified from mouse T cells and shown to act as an excellent substrate for rDot1p methylation of histone H3 (Figure 4). As with the yeast substrates, this activity disappeared when the mononucleosomes were treated with DNase I. This result indicates that histone H3 was directly methylated by Dot1p and did not require putative cofactors in the yeast nuclear extract that an evolutionarily conserved aspect of the nucleosome is required for Dot1p activity, and that histone H3 is not heavily methylated at Lys79 in mouse T cells. Together, these results suggest that rDot1p methylates histone H3 at Lys79 only in the context of the nucleosome and not when the histone is a free protein.

Lys79 of Histone H3 Is Critical for Silencing

To test if histone H3-Lys79 plays a direct role in silencing, strains containing the wild-type or the K79A histone H3 gene were examined for silencing of the *URA3* reporter gene. Replacement of wild-type histone H3 with the K79A allele resulted in a drastic loss of silencing at telomeres, *HML*α and *HMR*a, and a slight decrease in silencing at the rDNA (Figure 5A) without otherwise affecting cellular growth. Thus, histone H3-Lys79 has a critical function in gene silencing in yeast, suggesting that Dot1p controls silencing by methylation of histone H3-Lys79 and not by methylation of another unidentified substrate. The levels of Sir2p, Sir3p, and Sir4p were not affected in the *dot1*Δ and histone H3-K79A strains (Figure 5B), indicating that the loss of silencing was not caused by altered expression levels of the Sir genes.

Methylation of Histone H3-Lys79 by Dot1p Affects Binding of Sir Proteins to Telomeres

Dot1p, via its methylation of Lys79 on histone H3, might modulate silencing by changing the interaction of Sir proteins with nucleosomes, thus decreasing occupancy of Sir proteins at a silent locus. In order to begin to address this possibility, chromatin immunoprecipitation (ChIP) was used to assess the binding of Sir2p and Sir3p at silent loci.

In wild-type cells, consistent with earlier reports (Gotta et al., 1997; Lieb et al., 2001), Sir2p bound telomeres (the *URA3* gene inserted immediately adjacent to telomere VII-L), *HMR*a, and rDNA. Sir3p binding was similar to Sir2p with the exception that there was much less binding to the rDNA (Figure 5C and data not shown). In addition, both Sir2p and Sir3p bound to the weakly silenced subtelomeric Y′ elements. This subtelomeric element is present at about half of all the telomeres and is absent from the *URA3*-marked VII-L telomere. All of these interactions appeared to be specific, because neither Sir protein bound to any of these loci in a strain lacking *SIR2*, nor were they bound to two transcriptionally active loci (Figure 5C).

Deletion of *DOT1* resulted in a modest but reproducible decrease (~2-fold) of Sir2p and Sir3p binding to the marked telomere (Figure 5C). Similar results were found for a unique sequence adjacent to a subtelomeric X element at the endogenous VI-R telomere (data not shown). There was no detectable change in the binding of Sir2p at the rDNA and little or no reduction in Sir2p or Sir3p binding at *HMR*a. In contrast, there was a modest *increase* in Sir2p and Sir3p binding at the subtelomeric Y′ elements (~2-fold). The effects on Sir protein cross-linking correlate with the effect on gene expression at each locus when *DOT1* is deleted (Hughes et al., 2000; Singer et al., 1998). Taken together, these results suggest that, in the absence of Dot1p-mediated Lys79 methylation of histone H3, the relative abundance of Sir proteins is redistributed between telomeric regions of the genome. Furthermore, they indicate that Dot1p is not absolutely required for Sir protein binding.

In order to evaluate the role of Lys79 per se on Sir binding, the same ChIP analysis was performed on a strain containing the histone H3-K79A mutation. The effect on Sir protein binding was more dramatic than, and in some ways distinct from, that observed in the *dot1*Δ strain. In the histone H3-K79A strain, Sir2p and Sir3p binding was reduced at all of the silent loci examined (Figure 5C), including the Y′ elements. The greatest reduction occurred at the marked telomere and Y′ elements (>10-fold), while *HMR*a and the rDNA were af-

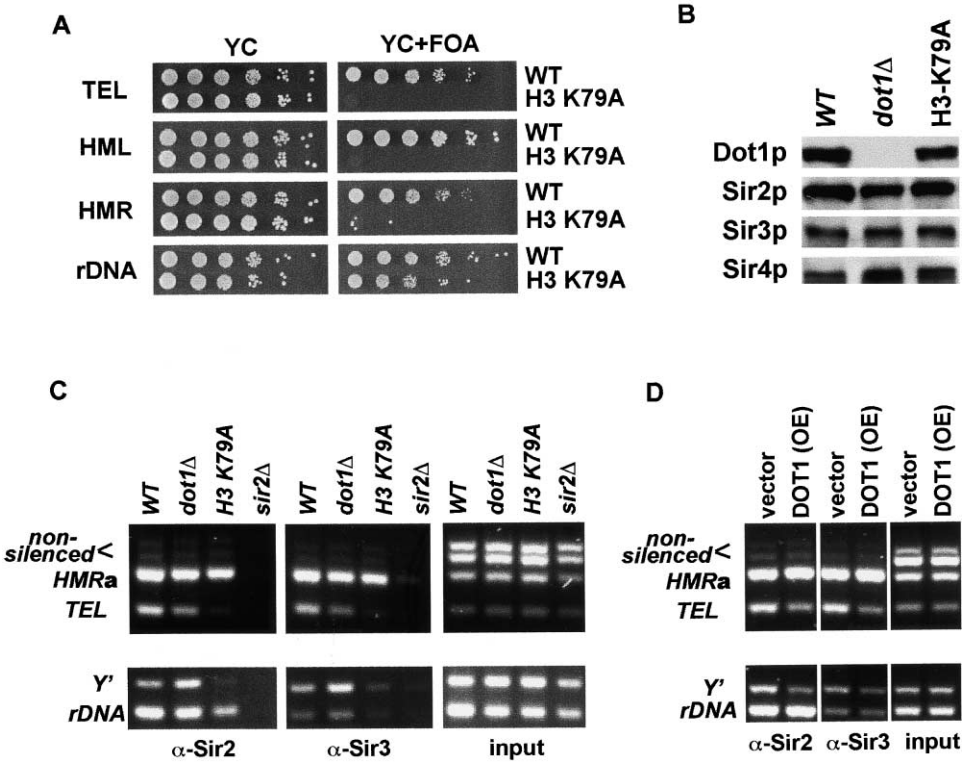

Figure 5. Methylation of Histone H3-Lys79 Regulates Targeting of Sir Proteins to Silent Domains

(A) Silencing of *URA3* at telomere VII-L (TEL), HML, HMR, and rDNA in wt and histone H3-K79A strains was measured as described in Figure 1.

(B) Steady-state Sir protein levels in strains UCC7201 (wt), UCC7202 (*dot1*Δ), and UCC7223 (H3-K79A) were determined by Western blot analysis.

(C) In vivo crosslinking analysis of Sir proteins was performed by ChIP using antibodies against Sir2p (α-Sir2) and Sir3p (α-Sir3). Sir protein binding was examined at the silent domains at the telomeric *URA3* gene (TEL), *HMR*a, and the rRNA 5S gene (rDNA), at the nonsilenced loci *GAL1* (top) and *SIR3* (bottom), and the subtelomeric Y′ elements (Y′). DNA of the input samples was amplified as a control (input). ChIP was performed on wt, *dot1*Δ, and H3-K79A (see B), and *sir2*Δ (UCC7095).

(D) As in (C), except wt cells (UCC1091) contained empty vector or the *DOT1* overexpression plasmid [DOT1(OE)] and were grown on selective media containing galactose (see Figure 1D).

fected more modestly (~2- fold). These results show that Lys79 of histone H3 is important for effective binding of silencing proteins to chromatin.

We also looked at the effect of hypermethylation of histone H3-Lys79 by overexpression of Dot1p, which disrupts silencing at *HMR*a, telomeres, and the rDNA (Singer et al., 1998). Upon overexpression of Dot1p, a modest decrease in Sir2p and Sir3p binding was observed at the marked telomere and Y′ elements (Figure 5D). At *HMR*a and the rDNA repeats, no difference in Sir protein binding was detected. Overall, this analysis revealed that histone H3-Lys79 is an important residue for Sir protein binding at silent domains and that changes in its methylation state can modulate binding and the targeting specificity of Sir2p and Sir3p.

Discussion

Dot1p Is a New Type of Histone Methyltransferase

In this study, we provide evidence that Dot1p methylates Lys79 of histone H3 in vitro and in vivo. Consequently, Dot1p joins other recently identified HMTs, which include the SET domain family of Lys HMTs and the PRMT family of Arg HMTs (reviewed in Zhang and Reinberg, 2001). Besides the difference in protein sequence, Dot1p has two striking biochemical properties that distinguish it from these other HMTs.

First, Dot1p methylates Lys79 in the core domain of histone H3, which contrasts with previously identified HMTs that modify residues in the NH_2-terminal tails of histone H3 or H4. One outcome of this difference may be that Dot1p is not regulated like these tail-specific enzymes. For instance, modification of residues in the NH_2 tail can enhance or prevent further modification of residues nearby (reviewed in Jenuwein and Allis, 2001; Zhang and Reinberg, 2001). Given that Lys79 is not in close proximity to the NH_2 tail modification sites and that Dot1p activity is unaffected by the absence of the histone H3 tail, it seems likely that Dot1p may define a pathway for regulating chromatin structure that is distinct from the information encoded in histone tails.

Second, previously identified HMTs, with the exception of the recently identified yeast Set2p (Strahl et al., 2002), act on purified histone protein or peptides of the histone tails in vitro, but have not been shown to act on histones packaged in chromatin (reviewed in Zhang and Reinberg, 2001). In contrast, we have not yet found Dot1p to be capable of methylating purified histone H3

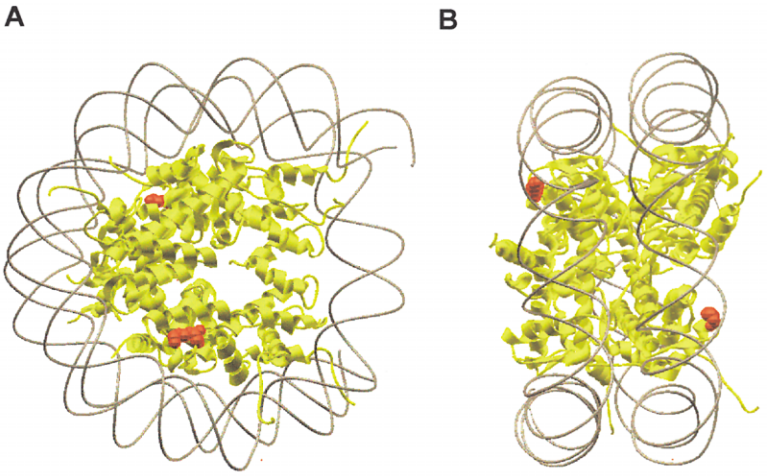

Figure 6. Lys79 on Histone H3 Is Located on the Outside of the Nucleosome

(A) The structure of the yeast nucleosome core particle, viewed down the superhelical axis. Lys79 (backbone and side chain) on loop 1 of histone H3 is shown in red space filling, while the histone protein main chains are represented as yellow ribbons. Gray lines indicate DNA.

(B) Side view of the nucleosome core particle, perpendicular to the view in (A). Figures were prepared using Swiss PDB Viewer and the coordinates from accession number 1ID3 in the Protein Data Bank (White et al., 2001).

protein; rather, Dot1p only methylated Lys79 in a chromatin context or in mononucleosomes.

Lys79 of Histone H3 Is Located at the Surface of the Nucleosome

The crystal structure of the nucleosome (Luger et al., 1997; White et al., 2001) sheds light on how Dot1p might gain access to Lys79 of histone H3 and what the implications of this modification are. Lys79 is located in the histone-fold domain of histone H3 in loop 1. The most salient aspect of this position is that, given the symmetry of the nucleosome, the Lys79 residues of the two histone H3 proteins reside on the top and bottom surfaces of the nucleosome disk (see Figure 6), readily accessible for interaction with Dot1p. This position may also explain why Dot1p recognizes Lys79 only in the context of chromatin and not as a free histone. The nucleosome-constrained form of histone H3, or Lys79 in proximity to DNA, may be required for Dot1p activity.

The location of Lys79 on the top and bottom surfaces of the nucleosome also suggests that its methylation may regulate interactions of the nucleosome with other proteins besides Dot1p, such as silencing proteins. While there is good evidence for Sir protein interactions with the NH_2 tails of histones H3 and H4 (Carmen et al., 2001; Georgel et al., 2001; Hecht et al., 1995), the Sir protein interactions may extend to the body of the nucleosome as well; Sir3p can interact with tailless nucleosomal arrays (Georgel et al., 2001). There are several other examples of silencing proteins and chromatin remodeling complexes that interact with the core of the nucleosome (Brehm et al., 2000; Guyon et al., 1999; Nielsen et al., 2001; Shao et al., 1999). These types of interactions might be important for the folding of nucleosomes into silent chromatin and may be modulated by Lys79 methylation.

Modification of Lys79 may also regulate interactions between nucleosomes. While there is still debate about how nucleosomes are compacted into higher-order chromatin structures (reviewed in Fajkus and Trifonov, 2001; Woodcock and Dimitrov, 2001), there is some evidence that the top and bottom of the nucleosome provide surfaces for internucleosomal interactions (Fan et al., 2002; Horn et al., 2002). In some cases, these interactions facilitate stacking of nucleosomes on top of one another (White et al., 2001). Thus, another ramification of Lys79 methylation may be a change in the ability of nucleosomes to organize into a more compact form of chromatin. The inherent ability of chromatin to become compact may be required for Sir protein binding and silencing.

Regardless of which model might be at work, a better understanding of the methylation of Lys79 will also require knowing whether mono-, di-, and trimethyl forms of this lysine have the same or different effects on potential interactions.

How Does Methylation of Lys79 on Histone H3 Modulate Gene Silencing?

In order to explain how Dot1p affects silencing, it is useful to review the *DOT1* overexpression and *dot1*Δ phenotypes. In the absence of Dot1p, there is no detectable methylation of Lys79, and silencing is strongly disrupted at telomere-proximal genes with a small decrease at the *HM* loci (Singer et al., 1998). However, a small *increase* in silencing is observed within the subtelomeric Y′ elements (Hughes et al., 2000). By ChIP, Sir2p and Sir3p binding were reduced somewhat at telomere-proximal genes, but increased at the Y′ elements (Figure 5C). Immunostaining of yeast mitotic nuclei with an α-Sir3p antibody is also informative. In wild-type cells, Sir3p is found in 5–8 foci that colocalize with the clustered telomeres, but in a *dot1*Δ cell, there are approximately 10-fold more Sir3p foci, which are distributed throughout the nucleus (San-Segundo and Roeder, 2000). Thus, it appears that Sir binding is not abrogated in *dot1*Δ cells, but rather that Sir proteins become less restricted as to which loci they can bind.

How might methylation of Lys79 affect Sir protein localization? It is unlikely that this modification is directly required for silent chromatin; rather it may be associated with active chromatin. Consider that $\sim$90% of histone H3 is methylated on Lys79 (Figure 3), yet only a small fraction of the yeast genome is silenced by Sir3p and/or Sir2p. We estimate that the silenced rDNA array, *HM* loci, and telomeric regions together account for $\sim$10% of the yeast genome. Furthermore, as noted above, a lack of Lys79 methylation does not prevent Sir protein

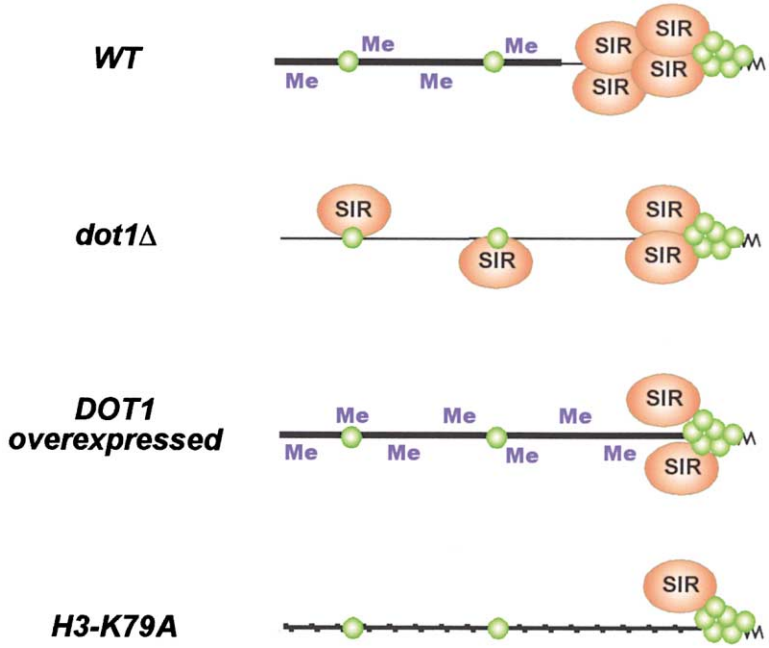

Figure 7. A Model for the Role of Histone H3-Lys79 Methylation by Dot1p in the Organization of Silent Chromatin

In wild-type (wt) cells, the Sir protein complex (SIR) is recruited to silencers at telomeres (and other silent domains) by specific DNA binding proteins (e.g., Rap1p, ORC, Abf1p, yKu, green spheres). At silent domains Sir proteins interact with histone H3 that is hypomethylated at Lys79 (thin line). Methylation of histone H3-Lys79 in bulk chromatin (Me, bold line) prevents the binding of SIR to chromatin at weak protosilencers (i.e., single binding sites for Rap1p, ORC, etc.; see text). In the absence of histone H3-Lys79 methylation (*dot1*Δ), SIR binding to chromatin becomes promiscuous. Sir proteins can bind to chromatin at weak proto-silencers, which results in reduced availability of Sir proteins for normally silent domains. Overexpression of *DOT1* results in hypermethylation of histone H3-Lys79, leaving little unmodified histone H3 in the nucleus. This leads to reduced Sir binding and a general loss of silencing throughout the genome. The histone H3-K79A mutation disrupts the interaction between chromatin and Sir proteins, resulting in a severe global loss of SIR-mediated silencing.

binding, but rather it changes the distribution of Sir proteins along the chromosome. Thus, it is more likely that Sir proteins bind to a subset of histone H3 that occurs in low abundance (e.g., histone H3 with Lys79 unmodified, or mono- or dimethylated), which we postulate is present within the small fraction of silent chromatin (Figure 7). Consistent with this idea, overexpression of Dot1p activity, which causes an increase in the overall level of Lys79 methylation (from ~90% to 98%) and a strong increase in the level of Lys79 trimethylation (from ~56% to 92%; Figure 3), reduces Sir binding at telomeres and Y′ elements (Figure 5D) and disrupts silencing (Figure 1).

In evaluating these results, it is important to appreciate the limits of ChIP. This technology can sensitively determine if a region of DNA is occupied by a protein within a population of molecules (or cells) and the relative number of DNA molecules that are occupied by the protein. However, the technology may be quite limited in determining how many protein molecules occupy that region of DNA. In other words, we suspect that there is not a strict correlation between the number of Sir proteins bound at a locus and the amount of cross-linked DNA that is coimmunoprecipitated. Thus, in the ChIP we performed, we believe the reduced PCR signals we detected for Y′ elements and telomeres indicate that many of these DNA sequences have no Sir proteins bound (or are below the level of Sir protein immunoprecipitation). At loci such as *HMR*a where we saw little or no change, it is impossible to determine whether there truly was no decrease in Sir protein binding or whether all molecules of *HMR*a DNA were still occupied but by fewer Sir proteins.

In principle, if Sir proteins bind preferentially to regions that lack Lys79 methylation, loss of Dot1p might allow the Sir proteins to bind promiscuously throughout the genome (see Figure 7). However, this relaxed specificity does not necessarily mean that Sir protein binding is completely random. Although the Sir complex interacts with the nucleosome, it is recruited to specific loci by DNA binding proteins such as Rap1p and ORC (reviewed in Gasser and Cockell, 2001). There are more than 500 ORC complexes and ~300 Rap1p molecules bound to DNA at loci throughout the yeast genome (Lieb et al., 2001; Wyrick et al., 2001). All of these are potential Sir complex recruitment sites that may form stable complexes in chromatin when Lys79 is not methylated. As further support for this idea, Sir proteins have been localized to a subset of these Rap1p and ORC binding sites (see supplementary material in Lieb et al., 2001; Wyrick et al., 2001), showing that even in wild-type cells these canonically nonsilenced sites have the potential to recruit Sir proteins. We suggest that one of Dot1p's roles in the cell is to prevent sticky chromatin proteins, such as the Sir proteins, from binding indiscriminately along the chromosome. Although these cryptic Sir protein recruitment sites may not normally be sufficient to form a silent domain, they may act as "sinks" for the Sir proteins in a *dot1*Δ strain and thus effectively reduce the Sir protein occupancy of normally silenced regions. The loci that would be particularly sensitive are those that display semistable silencing, such as telomeres. Silencing of the mating-type loci is very stable and is generally more resistant to genetic or environmental perturbations that disrupt silencing at telomeres (reviewed in van Leeuwen and Gottschling, 2002).

Why is there a detectable increase in silencing and Sir2p/Sir3p occupancy at Y′ elements when telomeres without Y′ elements show a reduction in silencing and Sir occupancy? Y′ elements are a complex mixture of (1) DNA sequences that initiate silencing (i.e., the terminal telomere tract TG_{1-3}), (2) insulators that block the spread

of silencing (subtelomeric antisilencing regions or STARs), and (3) protosilencers that normally cannot initiate silencing but facilitate its spread when in proximity to an initiating element (Fourel et al., 1999; Lebrun et al., 2001; Pryde and Louis, 1999). In the absence of Lys79 methylation, we speculate that the STARs lose their insulating capabilities and the protosilencers become more effective, helping to recruit Sir proteins to the Y′ element at the expense of normally silenced loci such as the non-Y′ telomeres.

Does Dot1p Have a Role in Chromatin State Memory?

Methylation is thought to be a very stable form of post-translational protein modification (reviewed in Jenuwein and Allis, 2001), and so far there is no evidence for enzymes that can remove methyl groups from lysines or arginines on histones. Therefore, methylation of Lys79 has the potential to serve as chromatin "memory," marking a locus as active, or nonsilenced, and preventing it from becoming silenced after chromatin duplication and cell division. If replicated DNA strands are assembled into chromatin by using a mixture of the local old (parental) histones and newly synthesized histones, the methylated parental histones may prevent the assembly of silencing proteins at the locus after cell division, and thus facilitate propagation of the nonsilenced transcriptional state. Conversely, if a silent region is replicated, unmethylated histones may allow the assembly of silencing proteins, preventing Lys79 from future methylation by Dot1p, thus facilitating propagation of the silenced state.

Because Dot1p acts only on chromatin and not on free histone H3 protein, it is uniquely suited to leave a heritable mark. Only parental histones, packaged in nucleosomes before DNA replication ensues, will be methylated, while newly synthesized free histones are immune to being marked. Interestingly, the *DOT1* gene is under transcriptional control, with peak expression at the G1/S transition (Spellman et al., 1998), which might provide a peak of Dot1p activity that could survey the genome for "open" chromatin and mark those nucleosomes as such before DNA replication takes place. Given that 90% of histone H3 in the cell is methylated in a wild-type cell and that Dot1p staining in the nucleus is diffuse (San-Segundo and Roeder, 2000), it seems unlikely that Dot1p is targeted to specific loci.

In summary, many studies have shown that post-translational modifications of the histone tails play an important role in gene silencing and other chromatin functions (Jenuwein and Allis, 2001). Here, we show that methylation in the core of histone H3, mediated by Dot1p, is also involved in gene silencing, and we propose that it helps limit silencing to specific loci by preventing the binding of Sir proteins elsewhere along the chromosome. This is not limited to mitotic cells, as *DOT1* plays a similar role to restrict the pachytene checkpoint proteins Sir2p and Pch2p on meiotic yeast chromosomes (San-Segundo and Roeder, 2000). Lastly, since both Dot1p and its substrate are conserved, we expect that methylation of histone H3-Lys79 plays a role in chromatin organization within most other eukaryotes.

Experimental Procedures

Yeast Strains and Methods

All strains were derived from BY4705 (Brachmann et al., 1998). UCC1369 (*MAT***a** *ade2::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh4::URA3-TEL-VIIL ADE2-TEL-VR hhf2-hht2:: MET15 hhf1-hht1::LEU2*, pMP9) was constructed in several steps to integrate *URA3* near telomere VII-L and *ADE2* near telomere V-R (van Leeuwen and Gottschling, 2002) and to replace the *HHF1-HHT1* and *HHF2-HHT2* loci with *LEU2* and *MET15*, respectively, in BY4705a (Kelly et al., 2000). UCC7120 and UCC7121 were generated by PCR-mediated gene replacement of *DOT1* by KanMX4 (Brachmann et al., 1998) in UCC1369 and UCC1091 (Kelly et al., 2000), respectively. UCC1188 (*MAT*α *leu2Δ1 lys2-801 trp1 ura3 hhf1-hht1::LEU2 hhf2-hht2::HIS3 RDN1::URA3*, pMP9) was generated from a cross between derivatives of JSS128 (Smith and Boeke, 1997) and RMY200U (Mann and Grunstein, 1992). UCC7262 (*MAT***a** *ade2 his3 leu2 lys2 ura3 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hmr::URA3,* pMP9) was generated from a cross between UCC1368 (congenic to UCC1369 except *MAT*α and no *URA3-TEL*) and UCC3513 (congenic to UCC3511 [Singer et al., 1998], except *MAT***a**). UCC7266 (*MAT***a** *ade2 his3 leu2 lys2 ura3 hhf1-hht1::LEU2 hhf2-hht2::MET15 ADE2-TEL-VR hml::URA3,* pMP9) was generated from a cross between UCC1369 and UCC3515 (Singer et al., 1998). The pMP9 plasmid in histone deletion strains was replaced with pMP3 (Kelly et al., 2000) or derivatives thereof to make the following strains UCC7201 (*URA3-TEL*), UCC7202 (*dot1*Δ, *URA3-TEL*), UCC7219 (H3Δ2-30, *URA3-TEL*), UCC7220 (*dot1*Δ, H3Δ2-30, *URA3-TEL*), UCC7223 (H3K79A, *URA3-TEL*), UCC7224 (*dot1*Δ, H3K79A, *URA3-TEL*), UCC7271 (*RDN1::URA3*), UCC7272 (H3K79A, *RDN1::URA3*), UCC7275 (*hmra::URA3*), UCC7276 (H3K79A, *hmra::URA3*), UCC7277 (*hml*α*::URA3*), and UCC7278 (H3K79A, *hml*α*::URA3*). UCC7095 was made by PCR-mediated gene replacement of *SIR2* by *HIS3* in UCC1369. Silencing assays were performed as described (van Leeuwen and Gottschling, 2002). *S. cerevisiae* transformation methods and media recipes are available (http://www.fhcrc.org/labs/gottschling/).

Plasmid Construction and Mutagenesis

The single-copy pLEU2-DOT1 vector contained a 5.5 kb genomic *Ssp*I fragment of the *DOT1* locus. pTCG-DOT1, a high copy *TRP1*-marked plasmid under the control of a *GAL1* promoter (Peterson et al., 2001), contained a full-length 1.8 kb *DOT1* fragment (−53 to +1807). Full-length *DOT1* was cloned into pET16b (Novagen) and expressed as a 10×HIS fusion in *E.coli*. Mutations were introduced by Quick Change (Stratagene) or by homologous recombination in yeast. Mutations and deletions in histone H3 were made in the *HHT2* gene in pMP3 (Kelly et al., 2000).

Histone Analysis and Methylase Assays

Yeast nuclear extracts were made similar to that described (Waterborg, 2000). No sodium butyrate was used. The washed nuclear pellet was resuspended in 0.3 ml sonication buffer (20 mM HEPES [pH 7.0], 200 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, and 5 mM β-mercaptoethanol) and then sonicated to solubilize chromatin (DNA size ranging from 0.2–2 kb). The nuclear extract was cleared by centrifugation for 30 min at 14,000 rpm at 4°C. Histones from nuclear extracts or MT assays were extracted and purified as described (Waterborg, 2000). Proteolytic peptides were fractionated by RP-HPLC between 0% and 40% acetonitrile (v/v) in 0.1% trifluoroacetic acid (v/v). For MS analysis, histone H3 protein was lyophilized and digested with Endoproteinase Arg-C (Roche) according to the manufacturer's instructions or with 100 ng trypsin (Worthington) in 50 mM ammonium bicarbonate at 37°C. Under these conditions, trypsin did not cleave the peptide bond at Lys79 of histone H3. Mononucleosomes were isolated from mouse T cell lymphoma EL4 (ATCC TIB-39) as described (Mizzen et al., 1999). Recombinant 10×HIS-tagged rDot1p was expressed in *E. coli* BL21(DE3)pLysS (Novagen) and purified using Talon metal affinity resin (Clontech) as described in the manual. In vitro methylation reactions were carried out in 10 mM Tris, (pH 7.9) using 1–5 μg of purified 10×HIS-rDot1, 0.1–5 μg substrate, and 1–2 μCi [^{3}H-methyl]-S-adenosyl-methionine (^{3}H-AdoMet) (78 Ci/mmol, 0.55–0.75 mCi/

ml), in a final volume of 15 μl and incubated for 0.5–6 hr at 30°C. Reactions were terminated by adding 5 μl 4× SDS PAGE loading buffer. Radiolabeled proteins were separated by SDS PAGE, transferred to PVDF membrane, and detected by autoradiography using intensifying screens (Kodak). For large-scale methylation assays, the reactions were scaled up to 1.5 ml, and for nonradioactive methylation assays cold AdoMet (20 mM stock in 10 mM H_2SO_4:ethanol 9:1) was added to a final concentration of 30 μM. Histone proteins and peptides were analyzed by LC-ESMS as described (Gatlin et al., 1998). After the peptides were identified, the data was used to quantify the relative methylation states of the histone H3 peptide 73-EIAQDFKTDLR-83. The ion current spanning 0.25 *m/z* on the high side and 0.25 *m/z* on the low side of the lowest isotopic peaks of the doubly charged unmodified, mono-, di-, and trimethylated histone H3 peptide 73-EIAQDFKTDLR-83 (*m/z* 668.5, 675.5, 682.4, and 689.6, respectively) were individually summed. Each ion current for a methylation state was divided by the sum of the ion current for all the methylation states to determine the relative abundances.

Antibodies and Chromatin Immunoprecipitation

A rabbit Dot1 polyclonal antiserum was made by immunization with the peptide CAARGRRNRGTPVKYTR conjugated to KLH. Mouse monoclonal Sir3 antibodies were made by immunization with baculovirus-expressed Sir3 protein. The Sir2 polyclonal goat antiserum was from Santa Cruz (Sc-6666). The Sir4 antiserum was a kind gift from D. Moazed. Polyclonal rabbit histone H3 tail antisera were from Upstate Biotechnology. ChIP was done as described previously (Dudley et al., 1999); primer sequences are available upon request. The amplified DNA fragments were separated by 2% agarose gel electrophoresis, stained with Vistra Green (Amersham), and analyzed with a PhosporImager (Molecular Dynamics).

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