A charge-based interaction between histone H4 and Dot1 is required for H3K79 methylation and telomere silencing: identification of a new *trans*-histone pathway

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Saccharomyces cerevisiae cells lacking Dot1 exhibit a complete loss of H3K79 methylation and defects in heterochromatin-mediated silencing. To further understand the mechanism of Dot1-mediated methylation, the substrate requirement of Dot1 was determined. This analysis found that Dot1 requires histone H4 for in vitro methyltransferase activity and the histone H4 tail for Dot1-mediated methylation in yeast. Mutational analyses demonstrated that the basic patch residues ($R_{17}H_{18}R_{19}$) of the histone H4 N-terminal tail are required for Dot1 methyltransferase activity in vitro as well as Dot1-mediated histone H3K79 methylation in vivo. In vitro binding assays show that Dot1 can interact with the H4 N-terminal tail via the basic patch residues. Furthermore, an acidic patch at the C terminus of Dot1 is required for histone H4 tail binding in vitro, histone H3K79 di- and trimethylation in vivo, and proper telomere silencing. Our data suggest a novel *trans*-histone regulatory pathway whereby charged residues of one histone are required for the modification of another histone. These findings not only provide key insights into the mechanism of Dot1 histone methylation but also illustrate how chromatin-modifying enzymes engage their nucleosomal substrates in vivo.

Histones have N- and C-terminal tails and histone fold domains that are subject to various post-translational modifications such as acetylation, phosphorylation, ubiquitination, and methylation (Vaquero et al. 2003; Mersfelder and Parthun 2006; Kouzarides 2007). These histone modifications can act as sites for recruiting other chromatin-associated factors (Maurer-Stroh et al. 2003; Kouzarides 2007; Ruthenburg et al. 2007). Together, histone modifications and the factors that bind to these modifications can influence the dynamic state of chromatin structure and regulate the chromatin environment and gene expression profiles of a cell.

In *Saccharomyces cerevisiae*, Set1, Set2, and Dot1 are histone lysine methyltransferases that methylate lysine residues in histones at H3K4, H3K36, and H3K79, respectively (Lee et al. 2005). Among the three sites of histone methylation, histone H3K79 is unique in that it is not located within the H3 N-terminal tail domain but in the histone core region. Specifically, this methylation occurs at the L1 loop of the histone fold domain that is exposed on the surface of the nucleosome (Luger et al. 1997; White et al. 2001). In addition, the yeast Dot1 and human Dot1L H3K79 methyltransferases are also unique in that they are the only histone lysine methyltransferases that do not have a SET domain. Instead, the catalytic core region of Dot1 and Dot1L shares sequence motifs that are similar to class I arginine methyltransferases (Dlakic 2001; Cheng et al. 2005).

Yeast Dot1 was initially identified as a high-copy disruptor of telomere silencing (Singer et al. 1998). However, loss of *DOT1*, mutations that disrupt the catalytic activity of Dot1, or mutations at histone H3K79 can all lead to loss of telomere silencing (Ng et al. 2002a; Park et al. 2002; van Leeuwen et al. 2002). In addition, H3K79 is abundantly methylated at euchromatin and is hypomethylated at heterochromatin (Ng et al. 2002a, 2003; van Leeuwen et al. 2002). Although the presence of H3K79 methylation in euchromatin seems counterintuitive to Dot1's silencing phenotype, it has been suggested

that the silencing defects caused by overexpression of Dot1, deletion of *DOT1*, or loss of H3K79 methylation are due to an indirect mechanism where protein factors necessary for direct heterochromatin silencing, such as Sir proteins (Sir2, Sir3, and Sir4), are titrated away from silent chromatin (van Leeuwen and Gottschling 2002). This is supported by the observation that deletion or overexpression of Dot1 leads to mislocalization of Sir proteins from heterochromatin (San Segundo and Roeder 2000; Ng et al. 2002a, 2003; van Leeuwen et al. 2002). In addition, loss of Sir proteins leads to increased H3K79 methylation at telomeres, and overexpression of Sir3 extends the heterochromatin region at telomeres and concomitantly reduces H3K79 methylation (van Leeuwen et al. 2002; Ng et al. 2003). Therefore, it is believed that a functional role for H3K79 methylation is to restrict Sir proteins at heterochromatin by preventing Sir proteins from nonspecifically binding euchromatin (van Leeuwen and Gottschling 2002).

Histone methylation in *S. cerevisiae* is also regulated by a *trans*-histone tail modification where ubiquitination of histone H2B by Rad6 is required for Set1-mediated H3K4 di- and trimethylation and Dot1-mediated histone H3K79 di- and trimethylation (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002; Shahbazian et al. 2005). However, Set1 and Dot1 can methylate core histones and recombinant nucleosomes in vitro that lack ubiquitylated H2B (Roguev et al. 2001; Santos-Rosa et al. 2002; Sawada et al. 2004). How H2B ubiquitination allows for Set1-mediated H3K4 and Dot1-mediated H3K79 di- and trimethylation in vivo is still unclear.

In this study, we have identified a *trans*-histone methylation event where the basic patch residues ($R_{17}H_{18}R_{19}$) of histone H4 are required for Dot1-mediated H3K79 methylation but not H3K4 or H3K36 methylation. We also identify an acidic region on Dot1 that is required for binding to the histone H4 basic patch, histone H3K79 di- and trimethylation, and proper telomere silencing. This

charge-based interaction between Dot1 and the H4 basic patch provides new mechanistic insights into Dot1-mediated H3K79 methylation and a novel *trans*-histone methylation event.

Results

Dot1 methyltransferase substrate specificity

Previous reports have shown that Dot1 prefers nucleosomal substrates in in vitro histone methyltransferase (HMTase) assays (Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002; Sawada et al. 2004), whereas methylation of core histones by Dot1 has not been observed (Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002; Sawada et al. 2004). To investigate the substrate specificity of Dot1, in vitro HMTase assays were performed using purified recombinant Dot1 with nucleosomes, core histones, or recombinant histones substrates. All in vitro HMTase assays were analyzed by filter-binding assays and scintillation counting to measure total S-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-SAM) incorporation as well as SDS-PAGE and fluorography to identify the specific histone labeled. As expected, Dot1 shows robust activity against nucleosomal substrates specific to histone H3 (Fig. 1A,B, lane 3). In addition to the robust activity of Dot1 on nucleosomal substrates, we did detect some incorporation of ^{3}H-Methyl on histone H3 when Dot1 was incubated with chicken core histones (Fig. 1A,B, lane 5) or human 293T core histones substrates (Fig. 1C, lanes 3,4). Dot1 was unable to methylate recombinant histone H3 (Fig. 1A,B, lane 7) or HPLC-purified H3 isolated from 293T cells (Fig. 1C, lane 5). These data suggest that other histones within the nucleosome and core histone mix can contribute to Dot1 activity on histone H3. To further test this idea, Dot1 methyltransferase activity was assayed using H3 and H4 separately or in combination as substrates (Fig. 1C). Sur-
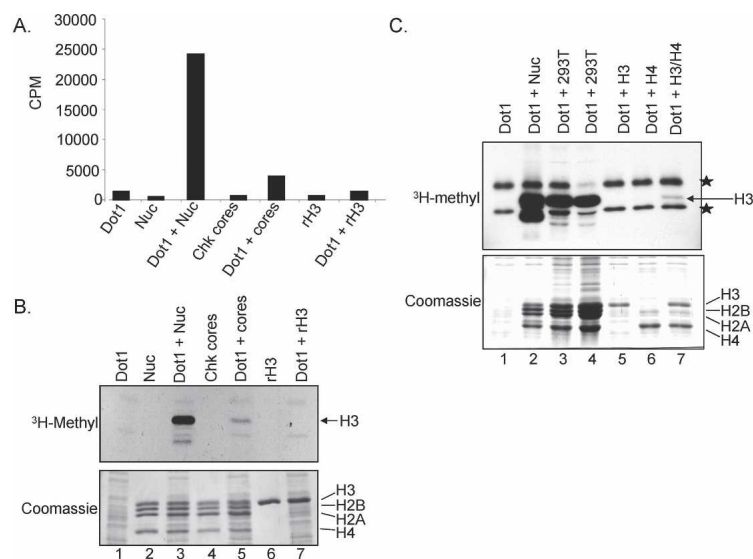

Figure 1. Dot1 methylates nucleosomes and core histones in vitro. (*A*) HMTase reactions were performed using recombinant-purified Dot1 incubated with ^{3}H-SAM and the following substrates: chicken oligonucleosomes (Nuc), chicken core histones (Chk cores), or recombinant histone H3 (rH3). Half of the reaction samples were analyzed by filter binding and scintillation counting. (*B*) The remaining reaction amounts (*A*) were resolved by SDS-PAGE and analyzed by Coomassie staining (*bottom* panel) and fluorography (^{3}H-Methyl, *top* panel). Positions of the individual histones are indicated. (*C*) Dot1 HMTase assays were performed with the following substrates: chicken oligonucleosomes (lane *2*), core histones from 293T cells (lanes *3,4*), HPLC-purified H3 or H4 from 293T cells (lanes *5,6*), and a mixture of HPLC-purified H3 and H4 (lane *7*). (*Top* panel) Samples were run on SDS-PAGE and fluorographed (^{3}H-Methyl). Two stars indicate the position of a nonspecific bacterial protein that was detected when overexposing the fluorographed gel. (*Bottom* panel) A Coomassie-stained gel of the HMTase assays is shown.

prisingly, Dot1 is able to weakly methylate H3 in a mixture of H3 and H4 histones (Fig. 1C, lane 7). Since it appears that Dot1 needs histone H4 to methylate H3, we wanted to further investigate how H4 participated in Dot1-mediated H3K79 methylation and to assess if a new *trans*-histone methylation event was occurring.

A basic patch of amino acids in histone H4 is required for H3K79 methylation

To further examine the apparent requirement of H4 for Dot1-mediated H3K79 methylation, we examined the histone methylation status of yeast strains expressing histone H3 and H4 N-terminal tail deletions. Yeast cells expressing wild-type or mutant histones were analyzed by Western blotting using antibodies that detect the various methylation states of H3 at K4, K36, and K79. Strikingly, global histone H3K79 di- and trimethylation is abolished in yeast strains expressing histone H4 lacking amino acids 4–19 (Fig. 2A, lane 2). Loss of H3K79 methylation is specific to deletion of the H4 N-terminal tail, as deletion of the N-terminal tail of H3 (amino acids 3–29) has no apparent effect on global H3K79 or H3K36 methylation (Supplementary Fig. S1, lane 3). Also, the H4 N-terminal tail deletion specifically results in loss of H3K79 di- and trimethylation but not H3K4 and H3K36 methylation (Fig. 2A, lane 2).

To further define the amino acids on histone H4 required for H3K79 methylation, we generated various H4 tail deletion mutants. This mutational analysis showed that amino acids 17–20, which correspond to the basic amino acids RHRK, is the region of H4 that is required for H3K79 methylation (Fig. 2A, lane 6). H3K79 monomethylation is greatly reduced in the H4$_{\Delta RHRK}$ basic patch mutant, while H4 Δ4–19 N-terminal tail deletion has intact monomethylation (Fig. 2A, lanes 2,6). These amino acids in H4 have been defined as part of the histone H4 basic patch (Johnson et al. 1990, 1992; Luger et al. 1997). In the crystal structure of the *Xenopus laevis*

nucleosome, these H4 basic patch residues interact with a patch of acidic residues contributed by histones H2A and H2B on an adjacent nucleosome (Luger et al. 1997). However, in the yeast nucleosome crystal structure, the H4 N-terminal tail forms a different conformation, poising the H4 basic patch to interact with the DNA of the neighboring nucleosome (White et al. 2001). Our data suggest the possibility of a new histone *trans*-tail pathway whereby the basic patch of the H4 N-terminal tail is required for H3K79 methylation.

Dot1 is not active on chromatin substrates lacking the H4 basic patch in vitro

Since there is a significant decrease in H3K79 monomethylation and no detectable H3K79 di- and trimethylation in yeast strains expressing the histone H4 basic patch mutant (H4$_{\Delta RHRK}$), we decided to examine if bacterially expressed Dot1 is active on yeast chromatin substrates lacking these residues. Soluble chromatin was isolated from the nuclei of yeast cells expressing the H4$_{\Delta RHRK}$ mutant or wild-type chromatin. In vitro HMTase assays were performed using these chromatin substrates incubated with purified recombinant Dot1 and ^{3}H-SAM. In support of our in vivo methylation data, when Dot1 is incubated with H4$_{\Delta RHRK}$ chromatin as a substrate, little HMTase activity is observed as compared with a wild-type chromatin substrate (Fig. 2B). As a control, both of these chromatin substrates were assayed with the yeast HMTase Set2, a H3K36-specific methyltransferase, in order to determine if the H4$_{\Delta RHRK}$ chromatin can serve as a viable substrate in an HMTase assay. As expected based on our in vivo H3K36 methylation results, purified recombinant Set2 can methylate wild-type or H4$_{\Delta RHRK}$ chromatin equally well (Fig. 2B). Together, these data suggest that H4$_{\Delta RHRK}$ chromatin can serve as a viable substrate for HMTase activity, and that the loss of H3K79 methylation in vivo and in vitro is specifically due to the absence of the basic patch of

Figure 2. A basic patch of amino acids in histone H4 is required for H3K79 methylation in vivo. (*A*) Western blots of whole-cell extracts using methyl-specific antibodies show the methylation status of H3K4, H3K36, and H3K79 in yeast cells expressing wild-type histones or cells expressing the indicated histone mutants. Antibodies directed against histone H3 and acetylated H4 serve as loading controls. Cells expressing mutant histones H3K4R, H3K36R, or H3K79R serve as negative controls for the indicated histone antibodies. (*B*) Dot1 is not active on yeast chromatin substrates isolated from cells lacking the basic patch of histone H4 in vitro. Recombinant-purified Dot1 (1.2 μg) was incubated with soluble chromatin substrates isolated from yeast cells expressing either wild-type histone H4, or from cells expressing histone H4 lacking the basic amino acids R17–K20 (H4$_{\Delta RHRK}$) in the presence of ^{3}H-SAM in an in vitro HMTase assay. Incorporation of ^{3}H-Methyl was measured by scintillation. Recombinant-purified Set2 (2.0 μg) was used as a control.
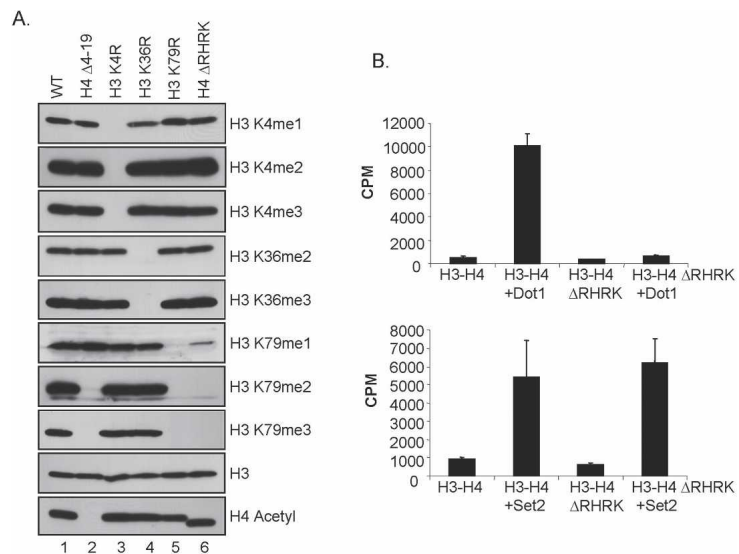
histone H4. Our in vitro results further suggest that Dot1 directly recognizes the H4 basic patch to mediate H3K79 methylation in vivo.

The basic amino acids of the basic patch of histone H4 are necessary for H3K79 methylation

After determining that the basic patch of histone H4 is necessary for H3K79 methylation, we performed additional mutational analyses to further characterize the role of this region in Dot1-mediated H3K79 methylation. Yeast strains were engineered to express mutant histones in which the basic amino acids in the basic patch of H4 have been substituted by neutral, acidic, or basic amino acids. Histone H4 double mutants were generated with both R17 and R19 changed to either neutral or acidic amino acids. Cells expressing these mutants were analyzed by Western blotting, and the methyl state of H3K79 was examined. Interestingly, wild-type levels of H3K79 methylation are detected only when the substitutions made at R17 ($H4_{R17K}$) and R19 ($H4_{R19K}$) maintained the positive charge (Fig. 3A, lanes 7,10). Histone $H4_{R17K,R19K}$ double mutants also have wild-type levels of H3K79 mono- and dimethylation, but the levels of trimethylation are slightly reduced (Fig. 3A, lane 13). Yeast strains expressing neutral charge mutants, $H4_{R17A}$ and $H4_{R19A}$, show near wild-type levels of H3K79 monomethylation but dramatically reduced or abolished amounts of H3K79 di- and trimethylation, respectively (Fig. 3A, lanes 5,8). Monomethylation is significantly re-

duced and di- and trimethylation of H3K79 are undetectable in the $H4_{R17A,R19A}$ and $H4_{R17E,R19E}$ double mutants (Fig. 3A, lanes 11,12). The reduction of H3K79 dimethylation in the single neutral amino acids substitution mutants is likely due to a partial reduction of the overall positive charge of this region of H4 (Fig. 3A, lanes 5,8). In contrast, H3K79 di- and trimethylation were completely abolished when H4R17, H4R19, or both residues were replaced by acidic amino acids (Fig. 3A, lanes 6,9,12).

Surprisingly, H3K79 monomethylation seems to be relatively unaffected in yeast with neutral amino acid substitutions at H4R17 or H4R19 (Fig. 3A, lane 5,8), similar to what is observed with the H4 Δ4–19 N-terminal tail deletions (Fig. 2A, lane 2). However, a $H4_{R17E}$ mutation appears to have a more dramatic impact on H3K79 monomethylation than a $H4_{R19E}$ mutation (Fig. 3A, lanes 6,9). In addition, $H4_{R17A}$ mutants have a more substantial impact on H3K79 dimethylation than $H4_{R19A}$ (Fig. 3A, lanes 5,8). Double substitutions at R17 and R19 to either neutral or acidic amino acids greatly reduce H3K79 monomethylation to similar levels as $H4_{R17E}$ mutants and $H4_{ΔRHRK}$ mutants, suggesting the charge contributed by R17 is more important for H3K79 methylation. In addition, yeast chromatin isolated from a $H4_{ΔRHRK}$ mutant also has detectable, but reduced, H3K79 monomethylation, yet lacks H3K79 di- and trimethylation (Fig. 2A; Supplementary Fig. S2A). No defects in histone H3K79 methylation are observed with H4K16 or H4K20 mutations (Supplementary Fig. S2D; data not shown). However, histone H4H18 mutations also lose histone H3K79 methylation (data not shown).
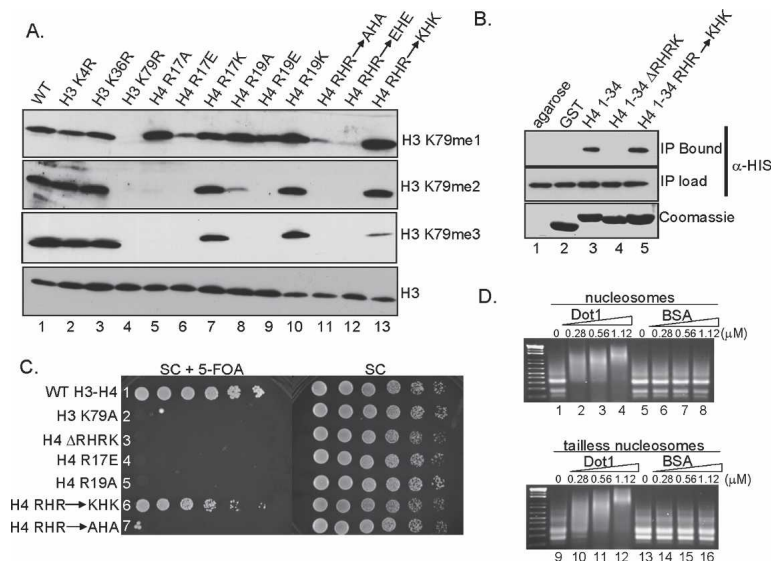

Figure 3. Basic amino acid residues within the basic patch of histone H4 are necessary for H3K79 methylation, Dot1 H4–tail interactions, and telomere silencing. (*A*) Western blots were performed using whole-cell extracts from cells expressing wild-type histones or the indicated histone mutations. The methylation status of H3K79 was examined using methyl-specific antibodies directed against H3K79me1, H3K79me2, and H3K79me3. Antibodies specific for histone H3 were used as a loading control. (*B*) In vitro binding assays were performed to test if Dot1 interacts with the H4 N-terminal tail. Bacterial cell extracts from cells expressing recombinant His_6-Dot1 were incubated in the presence of a GST-H4 tail encoding residues 1–34 of histone H4 (GST-H4$_{1–34}$; lane *3*), GST-H4$_{1–34}$ lacking the basic amino acids R17–K20 (GST-H4$_{1–34ΔRHRK}$; lane *4*), and GST-H4$_{1–34}$ containing R17K and R19K mutations (GST-H4$_{1–34RHR–KHK}$; lane *5*) bound to glutathione agarose beads. Bound His_6-Dot1 was detected by α-HIS antibodies. (*Top* panel, lane *2*) GST-bound beads or glutathione agarose beads alone were incubated with Dot1 extracts as negative controls. (*Middle* panel) Reaction inputs were probed with α-HIS antibodies to confirm equivalent amounts of Dot1 (IP load). GST-histone constructs were Coomassie-stained to indicate the amount of GST histone fusion protein loaded per lane. (*C*) Maintenance of charge of the H4 basic patch is required for telomere silencing. Strain UCC1369 (*URA3-TEL-VIIL*), expressing the indicated histone mutations, was grown to saturation, normalized to OD_{600}, serially diluted (4×), and spotted on SC or SC + 5-FOA media. UCC1369 expressing wild-type histones and H3$_{K79A}$ served as controls for telomere silencing assays; cells were grown on SC media as a growth control. (*D*) Nucleosome-binding gel mobility assays were performed using chicken erythrocyte nucleosomes and recombinant-purified His_6-Dot1. Wild-type nucleosomes (lanes *1–8*) and tailless nucleosomes (lanes *9–16*) were used as substrates. BSA was used as a control. The concentration of purified proteins in each reaction is indicated.

Together, these results suggest that maintenance of a small basic patch involving amino acid residues $R_{17}H_{18}R_{19}$ in histone H4 is critical for Dot1-mediated H3K79 methylation.

Dot1 binds to the basic patch of histone H4 in vitro

The basic patch of histone H4 has been shown to be an important binding site for various proteins within yeast, including Iswi2 and the heterochromatin silencing proteins, Sir3 and Sir4 (Hecht et al. 1995; Fazzio et al. 2005). Therefore, we propose that Dot1 must directly interact with the basic patch of histone H4 to mediate histone H3K79 methylation.

To test this hypothesis, we constructed histone H4 N-terminal peptide GST fusion proteins, either coding for GST-H4$_{1-34}$ or GST-H4$_{1-34\Delta RHRK}$. These constructs were expressed in *Escherichia coli* and purified with glutathione agarose. *E. coli* extracts expressing recombinant His$_6$-Dot1 were incubated with either GST-H4$_{1-34}$ or GST-H4$_{1-34\Delta RHRK}$ fusion proteins bound to glutathione agarose. After removal of unbound lysates and washing, bound proteins were eluted with SDS sample buffer. Western blots using α-HIS antibodies were used to detect the presence of His$_6$-Dot1. As predicted, we observe that Dot1 binds to GST-H4$_{1-34}$ in this assay (Fig. 3B, lane 3) but not to GST or agarose beads (Fig. 3B, lanes 1,2). Furthermore, Dot1 is no longer able to bind to GST-H4$_{1-34\Delta RHRK}$, suggesting that Dot1–H4 tail interactions are mediated by this basic region of histone H4 (Fig. 3B, lane 4).

To determine if the H4–Dot1 interaction is charge-dependent, we constructed a GST-H4$_{1-34}$ fusion protein in which the amino acids H4R17 and H4R19 were substituted with lysine residues, thereby maintaining the positive charge. In our assay, Dot1 is still able to interact with a histone H4 mutant maintaining the charge status of the histone tail (Fig. 3B, lane 5), suggesting that Dot1 must interact with the basic amino acids of histone H4 to mediate histone H3K79 methylation.

The basic patch of histone H4 is important for telomere silencing

Previous reports have shown that Dot1 and H3K79 methylation are important for telomere silencing (Singer et al. 1998; Ng et al. 2002a; van Leeuwen et al. 2002). Interestingly, yeast cells expressing histone H4 basic patch mutations also have silencing defects in the mating-type loci (Johnson et al. 1990, 1992). Since Sir3 and Sir4 can bind to the basic patch of histone H4, it is believed that these mutations prevent Sir3 and Sir4 from binding and lead to loss of silencing (Johnson et al. 1990, 1992; Hecht et al. 1995).

To determine if the histone H4 basic patch is important for telomere silencing, we generated various histone mutants in a yeast strain containing an integrated *URA3* gene at *TEL-VII-L* (UCC1369, kindly provided by D. Gottschling). Wild-type and histone mutants were plated on media with the toxic uracil analog, 5-fluoroorotic acid (5-FOA). In a wild-type cell, the *URA3* gene, when placed at the telomere, is silenced, and cells will be able to grow on 5-FOA-containing media. In contrast, in cells that lose silencing, the *URA3* gene will be expressed, and cells will not grow and will die in the presence of 5-FOA. Strains expressing the various histone mutants were grown to saturation, normalized for OD$_{600}$, serially diluted, and spotted on either SC plates as a control or SC plates containing 5-FOA. We observe that cells lacking the H4 basic patch (H4$_{\Delta RHRK}$, Fig. 3C, row 3) exhibit a telomere silencing defect when grown in the presence of 5-FOA, similar to that of a H3$_{K79A}$ mutant positive control (Fig. 3C, row 2). Additionally, histone H4$_{R17E}$ and H4$_{R19A}$ mutants also exhibit defects in telomere silencing (Fig. 3C, rows 4,5).

To determine if maintaining proper charge at the basic patch is important in telomere silencing, we tested similar mutants as described previously. By maintaining the positive charge with conserved mutations (H4$_{R17K,R19K}$), near wild-type levels of silencing are obtained (Fig. 3C, row 6). In contrast, cells expressing histone H4$_{R17A,R19A}$ mutants exhibit a telomere silencing defect (Fig. 3C, row 7). Although different H4 mutations were used, similar observations have been previously reported where maintaining charge at the histone H4 basic patch is important for mating-type silencing (Johnson et al. 1990, 1992). Our data suggest that the charge of the H4 basic patch must be maintained for proper histone H3K79 mono-, di-, and trimethylation as well as telomere silencing.

Dot1 binds nucleosomes independently of histone tails

In cells expressing H4 lacking the basic patch, we observe defects in H3K79 methylation. In addition, if the basic patch of H4 is deleted in the context of a H4 histone–peptide GST fusion, Dot1 no longer interacts with this fusion protein. To test whether Dot1–nucleosome interactions were affected by the loss of the H4 basic patch, nucleosomes lacking histone N-terminal tails were prepared by trypsin digestion (Supplementary Fig. S3B; Yang and Hayes 2004). This method has been reported to remove residues 1–23 of histone H4, residues 1–27 of histone H3, residues 1–42 of histone H2A, and residues 1–34 of histone H2B (Whitlock and Simpson 1977). Nucleosome-binding assays were performed using chicken erythrocyte nucleosomes or tailless nucleosomes incubated with purified recombinant Dot1 and analyzed by agarose gel electrophoresis followed by ethidium bromide staining (Min et al. 2003). We observed that Dot1 is able to bind and shift both nucleosomes and tailless nucleosomes (Fig. 3D). It did appear that there was a slight reduction in Dot1 binding to tailless nucleosomes as compared with wild-type (Fig. 3D, cf. lanes 2 and 10). From this data, we conclude that Dot1 activity, while dependent on the H4 tail, does not require the H4 tail for nucleosomal binding. As expected, in vitro methyltransferase assays show that there is little to

no Dot1 activity on these tailless nucleosomes (Supplementary Fig. S3A). This is similar to what is observed in HMT assays using histone H4$_{\Delta RHRK}$ chromatin substrates (Fig. 2B). These data also indicate that there must be other sites of interaction occurring between Dot1 and the nucleosome.

An acid patch on Dot1 is needed for interacting with the H4 basic patch and for histone H3K79 di- and trimethylation

Since maintaining the positive charge of the histone H4 basic patch is necessary for histone H3K79 methylation and telomere silencing, we predict that Dot1 might have a corresponding region that interacts with the H4 basic patch. To identify this region, Flag epitope-tagged C-terminal Dot1 deletion constructs were generated and expressed in yeast in cells lacking Dot1 under the control of the *ADH1* promoter (Supplementary Fig. S2B). Yeast whole-cell lysates were generated and analyzed for H3K79 methylation status by Western blot analysis. We observed that H3K79 methylation is rescued in yeast cells expressing wild-type Dot1 as well as a Dot1 C-terminal truncation mutant (pDot1$_{1-575}$) (Supplementary Fig. S2B, lanes 3,4). However, yeast cells expressing a Dot1 mutant that lacks an additional 20 amino acids at the C terminus (pDot1$_{1-555}$) have H3K79 monomethylation but are unable to rescue H3K79 di- and trimethylation (Supplementary Fig. S2B, lane 5). Based on structural analysis of yeast Dot1, this C-terminal region of Dot1 forms an exposed loop structure that would make it accessible for protein–protein interactions (Sawada et al. 2004). Upon further inspection of this region, we iden-

tified an acidic patch from amino acids 557–561 containing four acidic residues, EDVDE. To determine if this acidic patch on Dot1 is needed for H3K79 di- and trimethylation, we deleted the acidic patch (EDVDE) in both yeast and *E. coli* Dot1 expression vectors. Wild-type Flag epitope-tagged Dot1 or Dot1 lacking the acid patch (Dot1$_{\Delta EDVDE}$) were expressed in yeast cells under control of the *ADH1* promoter, and the status of H3K79 methylation was examined by Western blot analysis. Similar to the Dot1 C-terminal truncation mutant (pDot1$_{1-555}$), we observe a loss of H3K79 trimethylation and a near complete loss of H3K79 dimethylation, as compared with a wild-type strain and a *dot1* deletion strain expressing wild-type Dot1 (Fig. 4A). Interestingly, we do observe wild-type levels of H3K79 monomethylation in cells expressing the Dot1$_{\Delta EDVDE}$ mutant.

To test if Dot1$_{\Delta EDVDE}$ had in vitro methyltransferase activity, Dot1$_{\Delta EDVDE}$, wild-type Dot1, and Dot1$_{G401R}$, a known catalytically inactive Dot1 mutant, were expressed in bacteria, purified, and assayed for HMTase activity using chicken erythrocyte nucleosomes as substrates. As indicated in Figure 4B, Dot1$_{\Delta EDVDE}$ mutants show significant reduction in its activity against nucleosomal substrates as compared with wild-type Dot1. However, the Dot1$_{\Delta EDVDE}$ mutant has some weak activity specific to H3, as compared with the catalytically inactive Dot1$_{G401R}$ mutant as indicated by filter and gel analysis (Fig. 4B; data not shown). Since Dot1$_{\Delta EDVDE}$ is able to still monomethylate in vivo, we suspect this in vitro activity is likely specific for H3K79 monomethylation.

Finally, to determine whether the Dot1 acidic patch mediates its interaction with the N terminus of histone
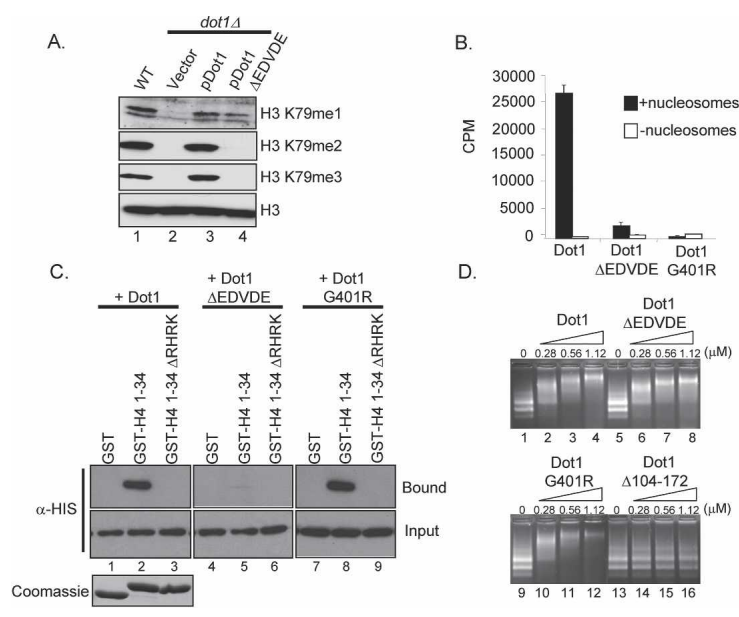

Figure 4. An acidic patch of amino acids in the C terminus of Dot1 is important for H3 methylation in vitro, K79 di- and trimethylation in vivo, and interaction with the H4 tail in vitro. (*A*) Western blots of whole-cell extracts using methyl-specific antibodies show the methyl status of H3K79 in wild-type or *dot1Δ* cells expressing full-length Flag epitope-tagged Dot1 or Dot1$_{\Delta EDVDE}$. Antibodies directed against histone H3 serve as a loading control. (*B*) In vitro methyltransferase assays using recombinant-purified His$_6$-Dot1, His$_6$-Dot1$_{G401R}$, or His$_6$-Dot1$_{\Delta EDVDE}$ incubated with chicken nucleosomes and ^{3}H-SAM. Incorporation of ^{3}H-Methyl was measured by scintillation. (*C*) GST-H4 peptide fusion pull-downs were performed as described previously. Recombinant-purified His$_6$-Dot1, His$_6$-Dot1$_{G401R}$, or His$_6$-Dot1$_{\Delta EDVDE}$ was incubated in the presence of a GST-H4 tail peptide fusion encoding residues 1–34 of histone H4 (GST-H4$_{1-34}$) or GST-H4$_{1-34}$ lacking the basic amino acids R17–K20 (GST-H4$_{1-34\Delta RHRK}$). (*Top* panel) Bound His$_6$-Dot1 and His$_6$-Dot1 mutants were detected by α-HIS antibodies (Bound). (*Middle* panels) Reaction inputs were probed with α-HIS antibodies to confirm equivalent amounts of Dot1 protein (Input). (*Bottom* panel) GST-histone constructs were Coomassie-stained to indicate the amount of GST histone fusion protein loaded per lane. (*D*) Nucleosome-binding gel mobility assays were performed using chicken erythrocyte nucleosomes and recombinant-purified His$_6$-Dot1, His$_6$-Dot1$_{G401R}$, His$_6$-Dot1$_{\Delta 104-172}$, or His$_6$-Dot1$_{\Delta EDVDE}$ at the indicated concentrations. Micromoles of Dot1 in each reaction are indicated.

H4, we performed in vitro binding assays in which GST-H4$_{1-34}$ was incubated with recombinant wild-type Dot1, Dot1$_{\Delta EDVDE}$, or Dot1$_{G401R}$. As compared with wild-type Dot1, binding of the Dot1 acidic patch mutant, Dot1$_{\Delta EDVDE}$, to GST-H4$_{1-34}$ is barely detectable (Fig. 4C, lanes 2,5). In addition, Dot1$_{G401R}$, although catalytically inactive, interacts with GST-H4$_{1-34}$, suggesting that Dot1 activity is not a prerequisite for H4 binding (Fig. 4C, lane 8). Together, our data support a model whereby the acidic patch of Dot1 must interact with the H4 basic patch for H3K79 di- and trimethylation.

Previously, Dot1 has been shown to bind nucleosomes, and that the N terminus of Dot1 is required for binding and methyltransferase activity (Sawada et al. 2004). To determine if the Dot1$_{\Delta EDVDE}$ mutant is able to bind nucleosomal substrates, nucleosomal binding assays were performed. As shown in Figure 4D, Dot1, Dot1$_{\Delta EDVDE}$, and Dot1$_{G401R}$ are all able to bind and shift nucleosomes as compared with lanes containing no Dot1 protein (Fig. 4D). As controls, a Dot1 mutant defective in nucleosome binding, Dot1$_{\Delta 104-172}$, and BSA were used (Fig. 4D, lanes 13–16; data not shown). Our results indicate that the Dot1$_{\Delta EDVDE}$ mutant is still capable of binding to nucleosomes, possibly to mediate monomethylation of H3K79. However, the nucleosome-binding ability of the Dot1$_{\Delta EDVDE}$ mutant is not sufficient to mediate di- and trimethylation of H3K79.

The acidic patch of Dot1 is important for telomere silencing

Previous studies have shown that yeast cells overexpressing Dot1 can disrupt telomere silencing (Singer et al. 1998; van Leeuwen et al. 2002). It has been shown that overexpression of Dot1 will cause H3K79 methylation to spread into regions of silent chromatin and displace proteins important for silencing (i.e., Sir3) away from telomere regions, resulting in silencing defects (Singer et al. 1998; van Leeuwen et al. 2002). This silencing defect is not observed if a catalytically inactive Dot1 is overexpressed (van Leeuwen et al. 2002). We wanted to determine if overexpressing Dot1$_{\Delta EDVDE}$, which lacks the ability to di- and trimethylate H3K79 and bind to the H4 N-terminal tail, would phenocopy the catalytically inactive Dot1 in telomere silencing assays. A yeast strain (UCC3503, kindly provided by D. Gottschling) expressing wild-type Dot1 and containing an integrated URA3 gene at TELVII-L was transformed with a plasmid encoding 3× hemagglutinin (HA$_3$)-tagged Dot1 (GAL-DOT1) or Dot1$_{\Delta EDVDE}$ (GAL-DOT1$_{\Delta EDVDE}$) under the control of a galactose-inducible promoter. Cells were grown in selective liquid media supplemented with glucose, normalized by OD$_{600}$, serially diluted, and spotted on selective media containing galactose or galactose and 5-FOA to monitor telomere silencing. Consistent with previous reports, we observe a telomere silencing defect when we overexpress Dot1 in a yeast strain expressing endogenous Dot1 (Fig. 5A, row 2; Singer et al. 1998; van Leeuwen et al. 2002). However, Dot1$_{\Delta EDVDE}$ overexpression did not result in a silencing defect in this strain, as growth on plates containing galactose and 5-FOA was similar to cells expressing vector only (Fig. 5A, cf. rows 3 and 1). Based on these data, Dot1$_{\Delta EDVDE}$ behaves as a catalytically inactive mutant with respect to telomere silencing, suggesting that H3K79 di- and trimethylation are important for silencing, whereas H3K79 monomethylation is not. Western blots show that Dot1 and Dot1$_{\Delta EDVDE}$ are equally expressed, and no global histone H3K79 methylation defects are observed in cells overexpressing Dot1 or Dot1$_{\Delta EDVDE}$ (Fig. 5B). Since the acidic region of Dot1 is important for binding to the basic patch of H4, loss or weakening of this interaction, as we have shown in vitro, may prevent overexpressed Dot1$_{\Delta EDVDE}$ from impacting silencing.

Dot1$_{\Delta EDVDE}$ was also tested for telomere silencing defects in strains lacking Dot1. Expression of HA$_3$-tagged

Figure 5. Dot1$_{\Delta EDVDE}$ overexpression does not result in a telomere silencing defect or detectable changes in H3K79 methylation in a strain expressing endogenous Dot1. (A) Yeast strain UCC3503 containing a plasmid encoding HA$_3$-tagged galactose-inducible Dot1 or Dot1$_{\Delta EDVDE}$ was tested for telomere silencing after galactose induction by plating on galactose containing media with or without 5-FOA. (B) Western blots were performed on whole-cell extracts after galactose induction to confirm that the HA-tagged Dot1 proteins were expressed upon induction. The methylation status of H3K79 upon Dot1 overexpression was also examined using H3K79 methyl-specific antibodies. (C) Cells expressing Dot1$_{\Delta EDVDE}$ exhibit a telomere silencing defect similar to a dot1Δ strain. Yeast strain UCC1369 expressing the indicated histone mutations and Dot1 constructs was examined for defects in telomere silencing. Cells were grown to saturation, normalized to OD$_{600}$, serially diluted, and spotted on selective media with or without 5-FOA.
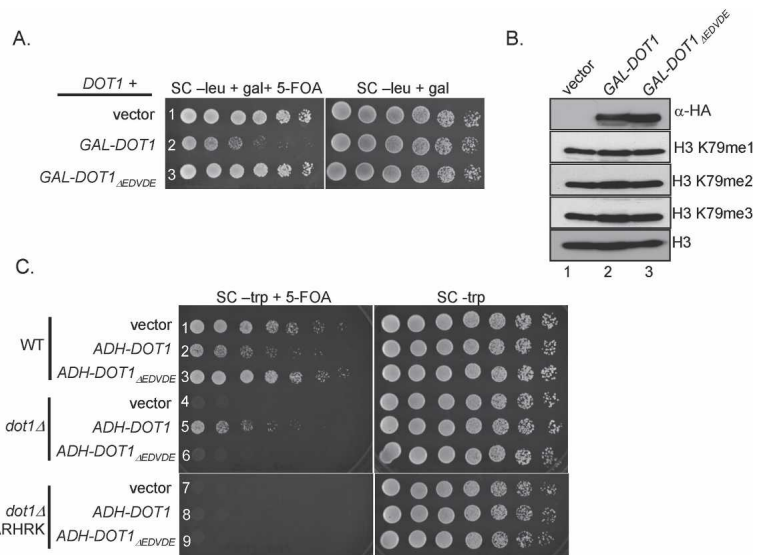
Dot1 (*ADH-DOT1*) from a constitutive *ADH1* promoter imparts a silencing defect similar to what is observed using galactose-driven Dot1 expression in a wild-type strain, but partially complements a *dot1*Δ mutant (Fig. 5C, rows 2,5). However, expression of HA$_3$-Dot1$_{\Delta EDVDE}$ (*ADH-DOT1$_{\Delta EDVDE}$*) does not complement a *dot1*Δ mutant (Fig. 5C, row 6). These results are in agreement with the *GAL-DOT1* and *GAL-DOT1$_{\Delta EDVDE}$* overexpression data (Fig. 5A). Furthermore, expression of Dot1 or Dot1$_{\Delta EDVDE}$ in a strain lacking endogenous Dot1 does not rescue the telomere silencing defect observed in a histone H4$_{\Delta RHRK}$ mutant strain (Fig. 5C, rows 8,9). Similar results are observed in cells expressing Dot1 or Dot1$_{\Delta EDVDE}$ using the endogenous Dot1 promoter (data not shown). These data support a model whereby the acidic patch of Dot1 interacts with the basic patch of histone H4. Therefore, the loss of this Dot1–H4 interaction prevents proper H3K79 methylation, resulting in the observed silencing defect.

Sir3 inhibits Dot1-mediated H3K79 methylation in vitro

Sir3 has been show to bind to the basic patch of histone H4, and in vivo results suggest a competition between Sir3 and Dot1 (Johnson et al. 1990; Hecht et al. 1995; van Leeuwen et al. 2002; Ng et al. 2003). Therefore, based on our observations that Dot1 can also bind to the histone H4 basic patch, it is possible that Sir3 and Dot1 compete for the same binding site. We tested this idea in an in vitro HMTase assay in which we examined Dot1 HMTase activity in the presence of increasing amounts of recombinant-purified Sir3 C terminus (amino acids 502–978) (Fig. 6). A similar C-terminal fragment of Sir3 has been shown to be sufficient for binding to the H4 N-terminal tail (Hecht et al. 1995). We observe that the presence of Sir3 can dramatically inhibit Dot1 activity, while the addition of a nonspecific competitor (BSA) showed no inhibition (Fig. 6). Based on our current data, we suggest that Sir3 displaces Dot1 from interacting with the H4 basic patch and thus prevents Dot1 from methylating nucleosomal substrates. Alternatively, since Dot1 and Sir3 can interact with tailless nucleosomes, Sir3 may globally displace Dot1 from a similar binding site within the nucleosome core, which would ultimately prevent Dot1 from interacting with the H4 basic patch. Nonetheless, we provide the first evidence of a direct mechanism of competition between Dot1 and Sir3, thus supporting known in vivo observations.

Discussion

In this report, we determined that Dot1 requires three basic residues (R$_{17}$H$_{18}$R$_{19}$) in the basic patch of histone H4 for H3K79 methylation. We also have determined that a charge-based interaction between an acidic domain of Dot1 and the basic patch of histone H4 is required for proper H3K79 methylation. Finally, we determined that the C-terminal acidic domain of Dot1 and
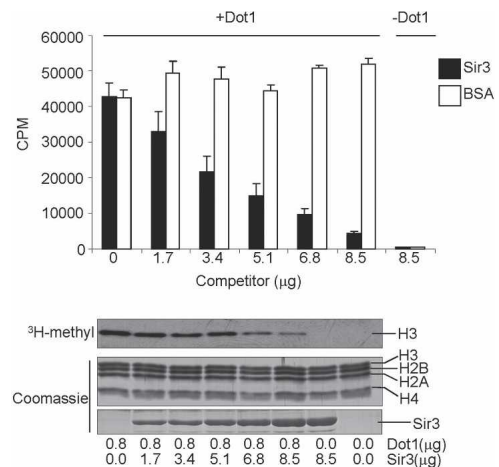

Figure 6. Sir3 inhibits Dot1-mediated methylation of nucleosomes. In vitro HMTase reactions were performed using recombinant-purified Dot1 incubated in the presence of increasing amounts of the recombinant-purified C terminus of Sir3 with nucleosomal substrates. Half of the reaction samples were analyzed by filter binding and scintillation counting. The remaining reaction amounts were resolved by SDS-PAGE and analyzed by fluorography (^{3}H-Methyl). (*Bottom* panels) Coomassie-stained gels show the protein inputs in each reaction. The amounts of purified protein in each reaction and positions of the individual histones are indicated.

the maintenance of the appropriate charge of the histone H4 basic patch are both required for telomere silencing. Altogether, we have identified a new *trans*-histone pathway that uses a charge-based mechanism to specifically facilitate histone H3K79 methylation.

Trans-*histone pathways*

Until now, only one other *trans*-histone pathway affecting histone lysine methylation has been identified in budding yeast. In *S. cerevisiae*, loss of histone H2B mono-ubiquitination or Rad6, the ubiquitin-conjugating enzyme that catalyzes this modification, results in a loss of H3K4 and H3K79 di- and trimethylation (Briggs et al. 2002; Dover et al. 2002; Ng et al. 2002b; Sun and Allis 2002; Shahbazian et al. 2005). Monomethylation of H3K4 and H3K79 appears to be unaffected in these mutants (Shahbazian et al. 2005). It is still unclear how H2B ubiquitination allows methylation of H3K4 and H3K79 to proceed from a monomethylated to a di- and trimethylated state. It is interesting to note that in cells expressing particular H4 basic patch mutants, or expressing a Dot1 acidic patch mutant that no longer interacts with the basic patch of histone H4, H3K79 monomethylation is still present. Therefore, it is quite possible that histone H2B ubiquitination affects the nucleosome structure to allow Dot1 access to the H4 basic patch region. Subsequently, this may allow Dot1 to mediate di- and trimethylation of H3K79. It is also likely that additional HMTases in yeast and other eukaryotes will have mechanisms that require another histone for *trans*-methylation.

Dot1-mediated histone H3K79 methylation

Yeast Dot1 and human Dot1L were the first non-SET domain histone lysine methyltransferases to be identified that specifically modify nucleosomal substrates (Feng et al. 2002; Lacoste et al. 2002; Ng et al. 2002a; van Leeuwen et al. 2002). However, our Dot1 acid patch mutants are still capable of binding to nucleosomes, yet lack the ability to di- and trimethylate H3K79. This demonstrates that nucleosome binding alone is not sufficient to mediate di- and trimethylation of H3K79. Since we do not see dramatic defects in H3K79 monomethylation in cells expressing $Dot1_{\Delta EDVDE}$, it suggests that there are other regions of Dot1 that may be important for nucleosome interactions that are necessary for this modification. Furthermore, $H4_{\Delta RHRK}$ mutants show a reduction in H3K79 monomethylation, yet abolished di- and trimethylation, which also implies that there are other potential points of interaction between Dot1 and histone H4.

The H4 basic patch interactions with Dot1 may contribute to the ability of Dot1 to methylate H3K79, possibly through facilitating Dot1 and H3K79 interactions prior to successive methylation events. It is also worth noting that the H4 tail is in close proximity to the H3K79 methylation site in the nucleosome crystal structure (Luger et al. 1997). From the Dot1 structure and Dot1–nucleosome docking model proposed in Sawada et al. (2004), the C-terminal acidic patch of Dot1 forms an exposed surface that is favorable for protein–protein interactions. Additionally, it appears that the acidic patch of Dot1 is in close proximity to the H4 tail and may accommodate the H4 tail basic patch. Although more structural analysis is needed to determine how Dot1 engages the nucleosome, our results indicate there are multiple modes by which Dot1 can uniquely interact with the nucleosome that are either permissive for nucleosome binding or methyltransferase activity.

Yeast Dot1 and human Dot1L both methylate H3K79, but Dot1L contains a C-terminal extension not found in Dot1 (Feng et al. 2002; Min et al. 2003). By sequence analysis, it appears that Dot1L does not possess the critical acidic patch of Dot1 that interacts with the H4 basic patch. However, there are stretches of acidic amino acids in the C-terminal extension of Dot1L, suggesting that a similar mechanism could exist for Dot1L. In humans, histone H4K20 is a major site of methylation, while little to no H4K20 methylation is present in *S. cerevisiae* (Fang et al. 2002; Nishioka et al. 2002; Garcia et al. 2007). Therefore, human Dot1L may have evolved slightly differently to deal with this additional chromatin modification.

Histone H4 basic patch

Our results indicate that there are three main amino acids $(R_{17}H_{18}R_{19})$ in histone H4 that are critical for H3K79 methylation, as mutations in H4K16 or H4K20 do not disrupt H3K79 methylation (Supplementary Fig. S2D; data not shown). Intriguingly, H3K79 monometh-

ylation is greatly reduced in the $H4_{\Delta RHRK}$ basic patch mutant, while the H4 Δ4–19 N-terminal tail deletion has intact monomethylation. We believe this difference is because Arg 3 in the H4 N-terminal tail essentially replaces R19 in the H4 Δ4–19 deletion mutant. These results are consistent with the observation that cells expressing only single-arginine mutants in the basic patch region exhibit wild-type levels of monomethylation (Fig. 3A). In addition, it is possible that other unknown amino acids in the H4 tail are needed for monomethylation.

The histone H4 basic patch is a site of interaction for chromatin-associated proteins such as Sir3, Sir4, *Drosophila* ISWI, and yeast Isw2 (Hecht et al. 1995; Clapier et al. 2001, 2002; Fazzio et al. 2005). Therefore, other factors that bind to or modify residues around the H4 basic patch may modulate Dot1's function. However, yeast lacking Sas2, a H4K16 histone acetyltransferase, Sir1, Sir2, Sir3, or Sir4 do not show any changes in global H3K79 methylation (Supplementary Fig. S2C,E). It has also been demonstrated that the chromatin remodeling activity of ISWI and Isw2 also needs the same three amino acids $(R_{17}H_{18}R_{19})$ in the histone H4 basic patch that Dot1 requires for H3K79 methylation (Clapier et al. 2001, 2002; Fazzio et al. 2005). However, yeast cells lacking Isw1, Isw2, or Itc1, an Isw2 complex member, have wild-type levels of H3K79 methylation (Supplementary Fig. S2C). Further investigation is needed to determine if other protein factors, or unknown histone modifications, regulate Dot1 binding to the H4 basic patch.

Balance of Sir proteins in maintaining proper telomere silencing

Since H3K79 methylation was first observed to be highly enriched in euchromatin, it was unclear why deleting or overexpressing Dot1 disrupts heterochromatin silencing (Ng et al. 2002a, 2003; van Leeuwen et al. 2002). It has been proposed that loss or overexpression of Dot1 disrupts telomere silencing by an indirect mechanism, where protein factors necessary for direct heterochromatin silencing, such as Sir proteins (Sir2, Sir3, and Sir4), are titrated away from heterochromatin to euchromatin (van Leeuwen and Gottschling 2002). Our observation that Dot1 can interact with the basic patch of histone H4 suggests that Dot1 and Sir3 and/or Sir4 are likely competing for binding to the histone H4 basic patch. Furthermore, we demonstrate that Sir3 can effectively compete Dot1 activity from nucleosomal substrates in an in vitro HMTase assay (Fig. 6). This competition could explain why decreases in H3K79 methylation are observed when Sir3 is overexpressed and why Dot1 overexpression displaces Sir3, leading to silencing defects.

Interestingly, in the context of the nucleosome, histones H3 and H4 contribute residues to form a patch of amino acids surrounding H3K79 that are important for heterochromatin silencing (Park et al. 2002). It has been proposed that the region surrounding H3K79 is a direct site for Sir3 and/or Sir4 binding since mutations of these residues disrupt silencing specifically at telomeres and mating type loci (Park et al. 2002). This is also supported
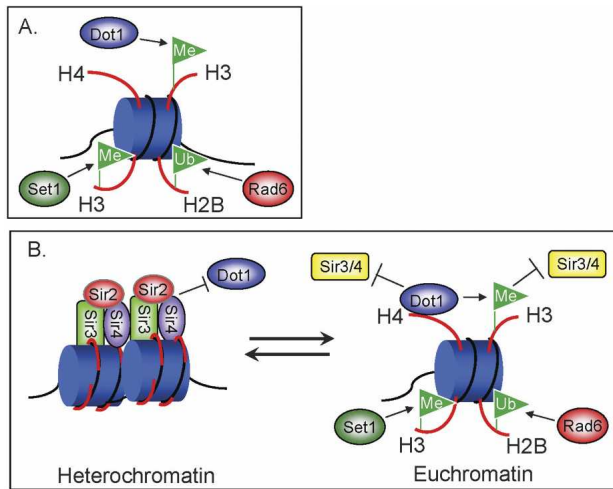
Figure 7. A newly identified *trans*-histone pathway. (*A*) A known *trans*-histone pathway important for histone methylation. Rad6-mediated ubiquitination of H2B required for H3K4 and H3K79 di- and trimethylation. (*B*) In heterochromatin, silencing is established and maintained by the presence of the Sir proteins (Sir2, Sir3, and Sir4). The presence of Sir3 and/or Sir4 can prevent Dot1 binding to the same target site on the histone H4 N-terminal tail, impeding Dot1 binding and H3K79 methylation at silent chromatin regions. In euchromatin, Dot1 interactions with the basic patch of the histone H4 N-terminal tail allows for H3K79 methylation, excluding the Sir3 and Sir4 proteins. It is likely that the presence of H3K79 methylation and the presence of Dot1 at active regions of chromatin both play a role in the exclusion of silencing factors at euchromatin.

by data showing that Sir3 can interact with nucleosomes lacking histone tails (Georgel et al. 2001). Therefore, H3K79 methylation may prevent Sir3 and/or Sir4 from binding to this region of the nucleosome surrounding H3K79. As illustrated in Figure 7, the presence of Sir3 and/or Sir4 at heterochromatin prevents Dot1 binding to the H4 basic patch and in turn prevents histone H3K79 methylation at the Sir3/Sir4 silenced heterochromatin. Conversely, at euchromatin, the presence of Dot1 prevents Sir3 and/or Sir4 from binding to the basic patch of histone H4. Dot1-mediated H3K79 methylation further excludes binding of Sir3 and/or Sir4, and thus antagonizes the spreading of heterochromatin (Fig. 7). Altogether, it appears that a delicate balance between proper levels of heterochromatin and euchromatin protein factors, mediated by charge-based interactions and histone modifications, is critical for the establishment and maintenance of these specialized chromatin regions in yeast.

Materials and methods

Yeast strains and plasmids

Plasmids and strains used in this study are listed in Supplementary Tables 1 and 2. See the Supplemental Material for details of plasmid and strain construction.

Yeast extract preparation, Western blotting, and antibodies

For histone analysis, yeast whole-cell extracts were prepared as described previously (Fingerman et al. 2005). Western blotting

to detect modified histones was performed as described previously (Briggs et al. 2001). Extracts were run on 15% SDS-PAGE gels, transferred to PVDF, and immunoblotted with the indicated histone antibodies (see the Supplemental Material for the histone antibodies used).

In vitro HTMase assays

In vitro HMTase assays were performed as described previously (Briggs et al. 2002; Milne et al. 2002) using recombinant-purified Dot1 (1.2 µg) or Set2 (2.0 µg) incubated with 6 µg of purified chicken nucleosomes and 1.0 µCi of ^{3}H-SAM. Yeast chromatin substrates were prepared as follows: Yeast strains expressing the desired histone mutants were grown to OD_{600} = 1.5–1.8, in a volume of 1 L in YPD medium. Cells were harvested and nuclei were prepared as described (Edmondson et al. 1996). To prepare soluble chromatin, 400 µL of nuclei/NP buffer suspension were centrifuged for 15 min at 5000g in a microfuge at 4°C to pellet nuclei. Supernatant was removed and nuclei were resuspended in sonication buffer (van Leeuwen et al. 2002) (20 mM HEPES at pH 7.0, 200 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, 5 mM β-mercaptoethanol) and sonicated to shear and solubilize the chromatin. The sample was then centrifuged at 14,000 rpm for 40 min at 4°C. The supernatant contained sheared, soluble chromatin. Sonicated chromatin samples were normalized, and 5 µg of each substrate were used in in vitro HMTase assays.

GST-H4-binding assays

BL21 cells expressing either GST-H4 tails (pGEX-2T $H4_{1-34}$, pGEX-2T $H4_{1-34\Delta RHRK}$, or pGEX-2T $H4_{1-34R17K, R19K}$) or His_6-Dot1 (pET28b-His_6-Dot1, His_6-$Dot1_{G401R}$, or His_6-$Dot1_{\Delta EDVDE}$) were grown to mid-log, induced with 0.4 mM IPTG for 4 h at 23°C, and harvested. Cell pellets were lysed by sonication at 4°C in 200 µL of lysis buffer (50 mM Tris-Cl at pH 8.0; 300 mM NaCl; 1 mM PMSF; 1 µg/mL each leupeptin, aprotinin, pepstatin). Cell lysates were clarified by centrifugation at 14,000 rpm for 5 min at 4°C. The supernatants contained soluble protein. For GST-H4 tail isolation, 10 µL of a 50% slurry of glutathione agarose (Sigma G4510) in PBS were added to the soluble fraction and incubated for 1 h at 4°C. After incubation, GST-H4 tail-bound agarose was pelleted and washed three times for 5 min each in lysis buffer. After washing, GST-H4 tail-bound beads were resuspended in 15 µL of lysis buffer. Of this final slurry, 1.5 µL was analyzed by SDS-PAGE for normalizing GST-H4 protein levels for binding reactions. Binding reactions were carried out as follows: Four microliters of GST-H4 tail-bound glutathione agarose beads were incubated with 20 µL of His_6-Dot1, His_6-$Dot1_{G401R}$, or His_6-$Dot1_{\Delta EDVDE}$ lysates in a final reaction volume of 200 µL, with final buffer conditions being 50 mM Tris-Cl (pH 8.0); 150 mM NaCl; 1 mM PMSF; and 1 µg/mL each leupeptin, aprotinin, and pepstatin. Ten microliters of each reaction were removed to be used as a control for Dot1 input. Binding reactions were incubated with rotation for 2 h at 4°C. Beads were washed with a modified RIPA buffer (50 mM Tris at pH 7.4; 75 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS; supplemented with 1 mM PMSF; 1 µg/mL each leupeptin, aprotinin, pepstatin) three times for 5 min each. After washing, beads were resuspended in 12 µL of 2× SDS-PAGE sample buffer. Samples were loaded onto a 12% SDS-PAGE gel and probed for His_6-Dot1 using a 1:5000 dilution of α-HIS antibody (Santa Cruz Biotechnology).

Telomere silencing assays

Telomere silencing assays were performed as described previously (Fingerman et al. 2005). Strain UCC3503 (see Supplemen-

tary Table 2 for genotype [Singer et al. 1998]) expressing the indicated histone was grown for 3 d to saturation in SC-Leu, normalized for OD_{600}, serially diluted (fourfold), and spotted (6 µL per spot) on SC-leu + galactose and SC-leu + galactose + 5-FOA (100 µg/mL) plates. Cell growth was monitored at 30°C over time. SC–galactose plates were photographed at 36 h, and SC–galactose–5-FOA were photographed at 48 h. Silencing assays using the ADH overexpression constructs were performed similarly, with the exception that cells were plated on SC-trp or SC-trp + 5-FOA (100 µg/mL).

Nucleosome-binding assays

Nucleome-binding assays were performed as described in Min et al. (2003). Briefly, equivalent amounts of recombinant-purified His_6-Dot1, His_6-Dot1$_{G401R}$, Dot1$_{\Delta104–172}$, or His_6-Dot1$_{\Delta EDVDE}$ were incubated with nucleosomes isolated from chicken erythrocytes (1.45 µM). Reactions were set up as for in vitro HMTase reactions, incubated for 1 h at 30°C, and resolved by agarose gel electrophoresis (2.0% agarose in 0.5× TBE). Gels were stained with ethidium bromide and visualized with UV light.

Preparation of tailless nucleosomes

Tailless nucleosomes were prepared as described in Yang and Hayes (2004). Briefly, isolated chicken erythrocyte nucleosomes were incubated with trypsin–agarose beads (Sigma). After the indicated times, the trypsin–agarose was removed by centrifugation. Tailless nucleosomes were confirmed by both Western blotting and SDS-PAGE, followed by Coomassie staining.

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