Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*

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Besides serving to package nuclear DNA, histones carry information in the form of a diverse array of post-translational modifications. Methylation of histones H3 and H4 has been implicated in long-term epigenetic 'memory'[1]. Dimethylation or trimethylation of Lys4 of histone H3 (H3 Lys4) has been found in expressible euchromatin of yeasts and mammals[2–4]. In contrast, methylation of Lys9 of histone H3 (H3 Lys9) has been implicated in establishing and maintaining the largely quiescent heterochromatin of mammals, yeasts, *Drosophila melanogaster* and plants[5–9]. We have previously shown that a DNA methylation mutant of *Neurospora crassa*, *dim-5* (defective in methylation), has a nonsense mutation in the SET domain of an H3-specific histone methyltransferase and that substitutions of H3 Lys9 cause gross hypomethylation of DNA[10]. Similarly, the KRYPTONITE histone methyltransferase is required for full DNA methylation in *Arabidopsis thaliana*[11]. We used biochemical, genetic and immunological methods to investigate the specific mark for DNA methylation in *N. crassa*. Here we show that trimethylated H3 Lys9, but not dimethylated H3 Lys9, marks chromatin regions for cytosine methylation and that DIM-5 specifically creates this mark.

We first characterized the specificity of recombinant DIM-5 using synthetic histone H3 N-terminal peptides (Fig. 1*a*). Consistent with the expectation that it is an H3 Lys9 methyltransferase, DIM-5 methylated an unmodified peptide (1–15) but not a similar trimethylated H3 Lys9 peptide. We found robust activity with a dimethylated H3 Lys9 peptide, which was a poor substrate for the H3 Lys9 methyltransferases SUV39H1 (ref. 5) and Clr4 (ref. 6). This suggested that DIM-5 efficiently methylates dimethylated H3 Lys9. We used Edman degradation to determine directly the site or sites of methylation on unmodified and dimethylated H3 Lys9 peptides (Fig. 1*b*,*c*). With both peptides, substantial incorporation occurred exclusively at Lys9. This confirms that DIM-5 is an H3 Lys9 methyltransferase and establishes that DIM-5 trimethylates H3 Lys9 *in vitro*.

We next addressed the possibility that DIM-5 might methylate additional residues, focusing on H3 Lys27 because G9a[12] and E(Z)[13,14] reportedly methylate both H3 Lys9 and H3 Lys27. Using an H3 peptide (21–34), G9a but not DIM-5 methylated H3 Lys27 (data not shown). To investigate whether DIM-5 methylates other positions, we compared its activity on recombinant H3 (1–57) with lysine or arginine at position 9. DIM-5 was only active on the unmodified polypeptide (Fig. 1*d*), suggesting that Lys9 is the only target in the H3 tail.

To further investigate the specificity of DIM-5, we used mass spectrometry to follow the kinetics of methyl transfer to unmodified or dimethylated H3 Lys9 peptides. With the unmodified substrate, DIM-5 produced all three methylation states early in the reaction (Fig. 1*e*,*g*). Trimethylated H3 Lys9 became the dominant form after 30 min, when there was still ample unmethylated substrate, and continued to increase relative to the other states. Notably, trimethylated H3 Lys9 was already dominant when dimethylated H3 Lys9 was first detected (2.5 min; Fig. 1*e*), implying that methylation of dimethylated H3 Lys9 is the fastest step catalyzed by DIM-5. Indeed, DIM-5 produced trimethylated H3 Lys9 much faster from dimethylated H3 Lys9 peptide than from unmodified peptide (Fig. 1*f*,*h*). These observations suggest that DIM-5 transfers methyl groups processively. The crystal structure of DIM-5 (ref. 15) shows that the methyl donor, AdoMet, is bound in an open pocket, which could permit the exchange of reaction product AdoHcy with AdoMet without releasing the substrate, permitting processivity. Alternatively, methylation may be distributive but DIM-5 simply prefers monomethylated and, especially, dimethylated substrates. In either case, we conclude that trimethylated H3 Lys9 is the main product of DIM-5 *in vitro*.

We investigated whether DIM-5 preferentially trimethylates H3 Lys9 *in vivo*. In preparation for chromatin immunoprecipitation (ChIP) analyses, we tested the specificity of antibodies generated with dimethylated and trimethylated H3 Lys9 peptides that have been used successfully in other systems[2,3,6,8,16–18]. Immunoblot analyses with unmodified H3 peptides or with dimethylated or trimethylated H3 Lys9 showed that the antibodies were highly specific for their respective epitopes (Fig. 2*a*), although the antibody against trimethylated H3 Lys9 cross-reacted weakly with the dimethylated H3 Lys9 peptide and less so with an unmodified peptide (see Supplementary Fig. 1 online). Given the similar sequence contexts of H3 Lys9 and H3 Lys27, we also tested whether the antibody against trimethylated H3 Lys9 cross-reacted with unmodified, dimethylated or trimethylated H3 Lys27. We found no evidence for recognition of H3 Lys27 (see Supplementary Fig. 1 online). We therefore used these antibodies and an antibody to dimethylated H3 Lys4 (a marker of euchromatin; refs. 2,3) to characterize the chromatin associated with methylated and unmethylated DNA regions.

Nearly all methylated sequences in the *N. crassa* genome seem to be relics of repeat-induced point mutation (RIP; refs. 19,20).

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We chose two representative methylated relics of RIP, *eta*[19] and *punt*[21], and two unmethylated genes, *pcn* and *hH4* (encoding proliferating cell nuclear antigen and histone H4, respectively; ref. 22) to analyze the methylation status of H3 Lys9. Southern-blot hybridizations using the isoschizomers *Dpn*II and *Sau*3AI showed cytosine methylation at *eta* and *punt* except in *dim-5* or *dim-2* strains, which are defective in histone[10] and DNA[23] methyltransferases, respectively (Fig. 2*b*). Because initial ChIP experiments suggested that *N. crassa* may lack dimethylated H3 Lys9, we used *Schizosaccharomyces pombe* chromatin as an internal positive control to detect dimethylated H3 Lys9. *S. pombe* strain SPG1355 (ref. 24) carries a small deletion (*ura4DS/E*) in the euchromatic endogenous *ura4* gene and an ectopic *ura4*$^{+}$ gene integrated into the heterochromatic *cen1* locus (*cen1:ura4*; characterized by dimethylated H3 Lys9 but not dimethylated H3 Lys4; refs. 3,6; Fig. 2*c*). We mixed cultures of SPG1355 and an *N. crassa* wild-type strain, fixed the chromatin with paraformaldehyde, immunoprecipitated it with various antibodies and carried out duplex PCR to compare methylated and unmethylated regions (*eta* and *pcn* or *punt* and *hH4*) of *N. crassa* and the *cen1:ura4* and *ura4DS/E* regions of *S. pombe*.

Notably, we found evidence of trimethylated, but not dimethylated, H3 Lys9 associated with the methylated DNA (Fig. 3*a*,*b*). As expected, the two unmethylated genes (*pcn* and *hH4*) were efficiently precipitated with the antibody against dimethylated H3 Lys4, whereas the inactive, methylated regions (*eta* and *punt*) were not. In contrast, the antibody against trimethylated H3 Lys9 preferentially precipitated both cytosine-methylated chromosomal regions. Similarly, we found relatively large amounts of trimethylated H3 Lys9 in a methylated *am* allele generated in the laboratory using RIP (*am*^{RIP8}; ref. 25; see Supplementary Fig. 2 online), supporting the idea that chromatin with methylated DNA is marked by trimethylated H3 Lys9 in *N. crassa*. The limited cross-reaction of the antibody against trimethylated H3 Lys9 with unmethylated H3 Lys9 (see Supplementary Fig. 1 online) accounts for the background reaction with non-methylated genes. We found no evidence for dimethylated H3 Lys9 in any methylated regions, whereas the *S. pombe* chromatin in the same samples showed dimethylated

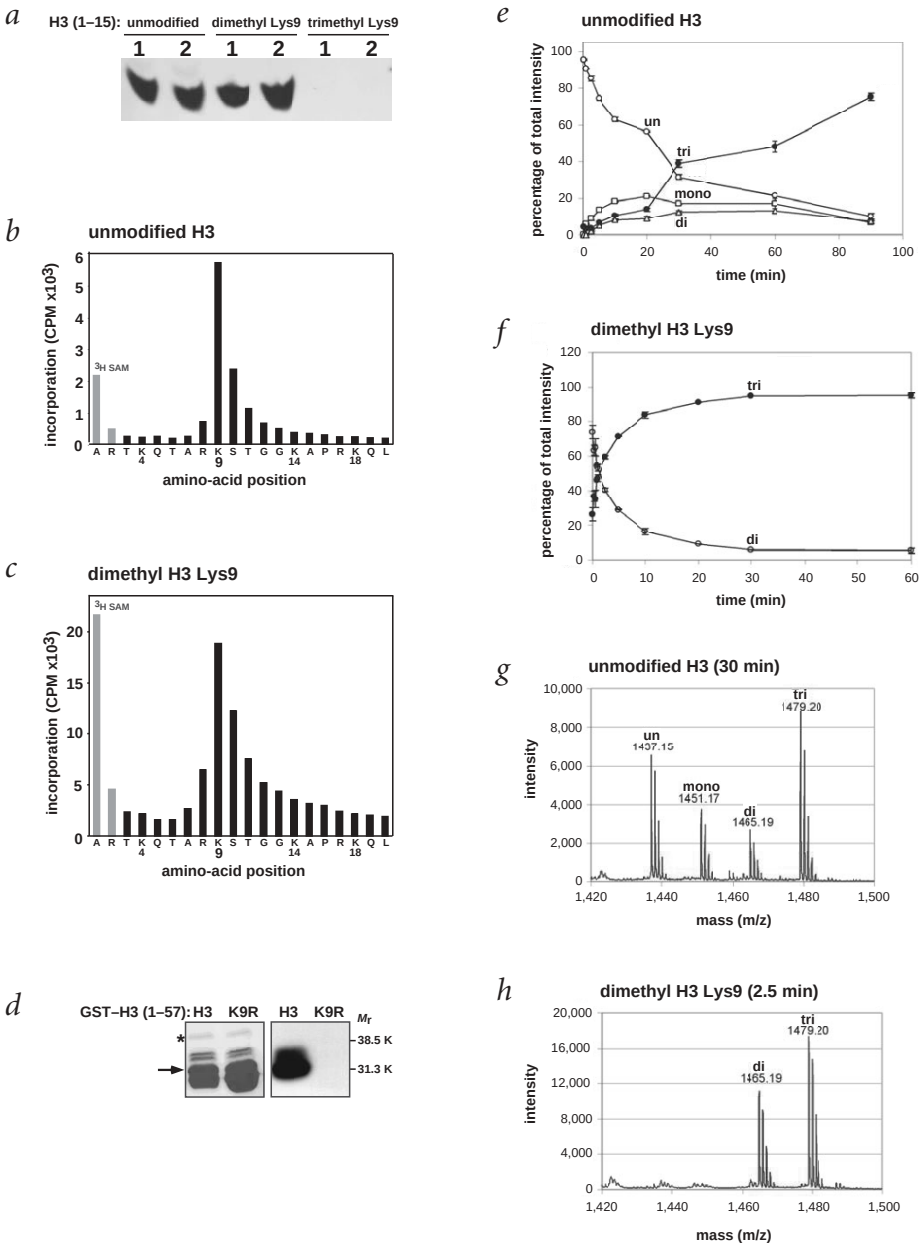

Fig. 1 DIM-5 trimethylates H3 Lys9 *in vitro*. ***a***, DIM-5 activity with unmodified, dimethylated, or trimethylated H3 Lys9 peptides (1–15). Each peptide was assayed independently twice (1 & 2). ***b***,***c***, Determination of amino-acid position of H3 peptides methylated by DIM-5. DIM-5 reactions were carried out as in *a* with either unmodified (*b*) or dimethylated (*c*) H3 Lys9 peptides (1–20). Reaction products were subjected to N-terminal sequencing, and incorporation of labeled methyl groups into individual amino-acid residues was detected by scintillation counting of each amino-acid fraction. The amino-acid sequence is shown below and lysine (K) residues are numbered. Fractions containing free ^{3}H AdoMet are indicated in gray. ***d***, DIM-5 activity with recombinant histone H3 tails (1–57) that were either unmodified or had a substitution of arginine[12] for lysine at position 9 (K9R) fused to glutathione S-transferase (GST–H3). Reaction products were fractionated by SDS–PAGE, stained with Coomassie blue (left) and then fluorographed (right) to detect methylation. A representative result of two independent assays with each peptide is shown. Positions of selected size standards, intact recombinant DIM-5 (asterisk) and GST–H3 fusion protein (arrow) are indicated. ***e***–***h***, Mass spectrometry analyses of DIM-5 products from unmodified or dimethylated H3 Lys9. DIM-5 reactions were initiated with unmodified (*e*) or dimethylated (*f*) H3 Lys9 substrate (3–15) and then stopped after incubation for the indicated times. Peptide masses were then measured. Relative intensity (%) of each mass was measured twice independently and plotted versus time. Mass identities of the H3 peptide with different Lys9 methylation status are indicated; un, mono, di and tri designate unmethylated, monomethylated, dimethylated and trimethylated H3 Lys9, respectively. Examples of mass profiles at 30 min with unmodified H3 peptide (*g*) and 2.5 min with the dimethylated H3 Lys9 peptide (*h*) are shown.

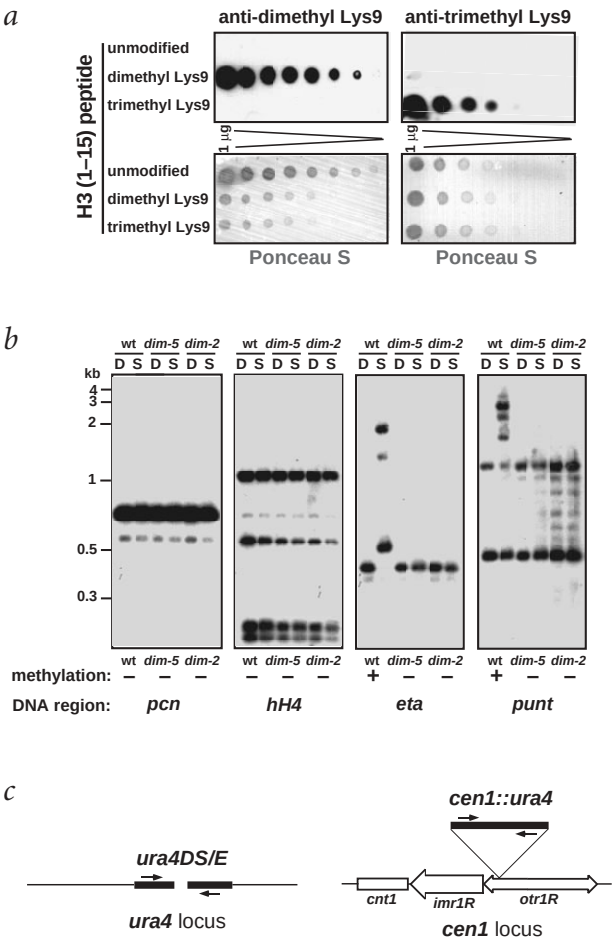

Fig. 2 Characterization of antibodies and genomic regions to analyze by ChIP. ***a***, Specificity of antibodies for methylated H3 Lys9. Starting with 1 μg of unmodified, dimethylated or trimethylated H3 Lys9 peptides (1–15), samples from a 2× dilution series were spotted onto a nitrocellulose membrane, stained with Ponceau S (bottom) and analyzed by immunoblotting (top) using antibodies against dimethylated or trimethylated H3 Lys9. ***b***, Southern-blot analyses of unmethylated (*pcn* and *hH4*) and methylated (*eta* and *punt*) chromosomal regions of *N. crassa*. Genomic DNA of the wild-type strain N150 (74OR23-IVA), *dim-2* strain N1877 (*dim-2:hph his-3 mat a*; ref. 23) and *dim-5* strain N2269 (*dim-5 mat A*)[10] was digested with *Dpn*II (D) or *Sau*3AI (S) and analyzed by gel electrophoresis and Southern-blot hybridization as described[30] using probes for the indicated regions. *Sau*3AI, but not *Dpn*II, is sensitive to cytosine methylation. The *pcn*, *hH4*, *eta* and *punt* probes were generated by PCR from the wild-type strain N150. ***c***, Diagram of endogenous euchromatic *ura4* allele carrying deletion (*ura4DS/E*) and an ectopic heterochromatic *ura4* allele integrated in *cen1* (*cen1:ura4*) in *S. pombe* strain SPG1355. PCR with primers *ura4DS/E*#1 and *ura4DS/E*#2 (ref. 24; indicated by arrows) generates products of distinctive lengths from *ura4DS/E* and *cen1:ura4*. The central component (*cnt1*) and part of the inverted repeats (*imr1R and otr1R*) of *cen1* are also represented.

H3 Lys9 at *cen1:ura4* (Fig. 3*c*) as expected[3,6]. Although these results suggest that H3 Lys9 is differentially methylated in silent regions of *N. crassa* and fission yeast, it is noteworthy that we detected enrichment of *cen1:ura4* relative to *ura4DS/E* with the antibody against trimethylated H3 Lys9. This could reflect trimethylation by Clr4 or by an unidentified histone methyltransferase. Considering that the same antibody against trimethylated H3 Lys9 paints heterochromatic regions in animals[18], it is unclear which methylation state of H3 Lys9 is most important in yeasts and animals. Our finding that trimethylation, but not dimethylation, of H3 Lys9 is associated with methylated *N. crassa* DNA is consistent with our observation that DIM-5 trimethylates H3 Lys9 efficiently *in vitro* and suggested that this mark may be critical for DNA methylation in *N. crassa*.

To determine whether DIM-5 is responsible for the trimethylation of H3 Lys9, we tested the effect of a nonsense mutation in the SET domain of DIM-5. We also examined the effect of a *dim-2* (DNA methyltransferase) mutation to test for feedback of DNA methylation on histone methylation (Fig. 4). Chromatin samples were immunoprecipitated with antibodies against trimethylated H3 Lys9, against dimethylated H3 Lys9 or against dimethylated H3 Lys4 and characterized by duplex PCR with all possible combinations of the two methylated (*eta* and *punt*) and two unmethylated (*pcn* and *hH4*) regions. With all four regions and all three strains, the antibody against dimethylated H3 Lys9 did not precipitate detectable chromatin, as before (data not shown). In contrast, the antibodies against trimethylated H3 Lys9 and against dimethylated H3 Lys4 preferentially precipitated the methylated and unmethylated regions, respectively, in the wild-type strain (Fig. 4*a*,*b*). The *dim-5* mutation markedly reduced the trimethylated H3 Lys9 signals in the methylated regions but did not reduce the weaker signal observed with the unmethylated genes. Our observation that the *dim-5* mutation did not increase H3 Lys4 methylation at *eta* or *punt* (Fig. 4*a*,*b*) indicates that H3

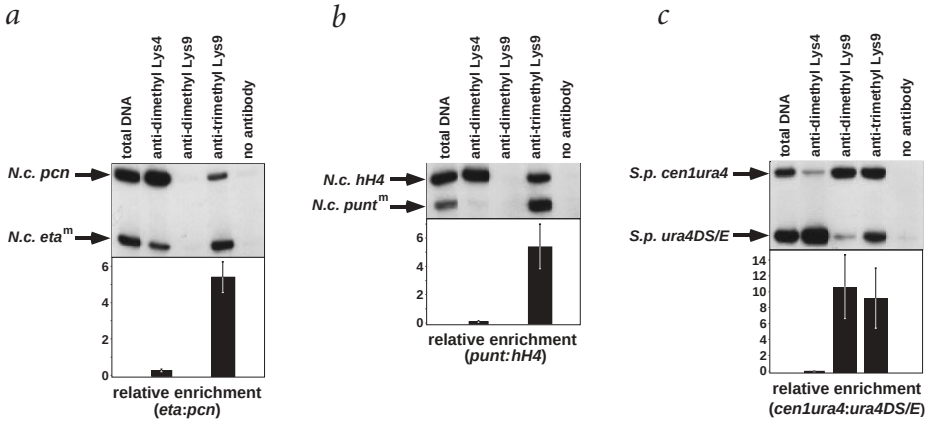

Fig. 3 Trimethylated, but not dimethylated, H3 Lys9 is associated with methylated DNA regions of *N. crassa*. Mixtures of extracts of *N. crassa* wild-type strain 74OR23-IVA and *S. pombe* strain SPG1355 were incubated with antibodies against dimethylated H3 Lys4, against dimethylated H3 Lys9 or against trimethylated H3 Lys9 (ref. 18) or were incubated without antibody. Total DNA, immunoprecipitated DNA and mock control DNA were subjected to duplex PCR to amplify pairs of unmethylated and naturally methylated (m) DNA regions of *N. crassa* (*a*,*b*) or a pair of heterochromatic (*cen1:ura4*) and euchromatic (*ura4DS/E*) *ura4* regions of *S. pombe* (*c*). Products were fractionated by gel electrophoresis and autoradiographed. No PCR product from *N. crassa* DNA was detected with antibody against dimethylated H3 Lys9 even after more PCR cycles (data not shown). Bar graphs below the PCR products represent enrichment of *eta* relative to *pcn* (***a***), *punt* relative to *hH4* (***b***) and *cen1:ura4* relative to *ura4DS/E* (***c***) with the indicated antibody. The relative enrichment was normalized to the ratios obtained from total DNA. Results from two PCR reactions from each of two independent ChIP experiments were averaged. Error bars represent s.d.

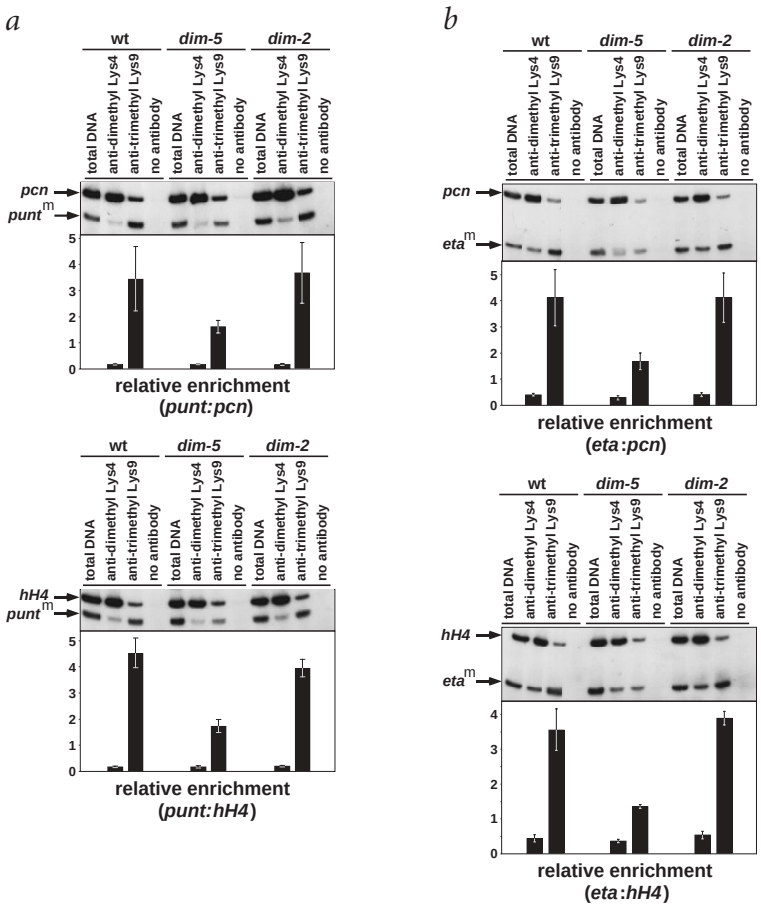

Fig. 4 DIM-5 is responsible for H3 Lys9 trimethylation associated with methylated DNA. ChIP experiments with chromatin from the *N. crassa* wild-type strain 74OR23-IVA, *dim-5* strain N2269 (*dim-5 mat A*; ref. 10) and *dim-2* strain N1877 (*dim-2:hph his-3 mat a*; ref. 23) were carried out using the indicated antibodies as described in Figure 3 but in the absence of the *S. pombe* chromatin. Duplex PCR was carried out to amplify (**a**) *pcn* and *punt* (top), *hH4* and *punt* (bottom) (**b**) *pcn* and *eta* (top) or *hH4* and *eta* (bottom) from the DNA samples. Enrichment of *punt* (*a*) and *eta* (*b*) relative to *pcn* or *hH4* are represented as in Figure 3.

Lys4 hypomethylation in the cytosine-methylated regions does not simply reflect competition between DIM-5 and an H3 Lys4 methyltransferase. DIM-5 is responsible for most, if not all, of the trimethylated H3 Lys9 in the methylated regions but not for the lower signal observed in the unmethylated regions. The residual signal probably represents cross-reaction of the antibody with unmethylated H3 Lys9 or another unidentified epitope. Mutation of *dim-2* did not reduce trimethylated H3 Lys9 at *eta* and *punt*, consistent with the conclusion that DNA methyltransferase acts downstream of DIM-5. Nevertheless, the *dim-2* mutation caused less H3 Lys9 trimethylation in a cytosine-methylated region (am^{RIP8}) that is transcribed when demethylated (ref. 26; see Supplementary Fig. 2 online). Thus, DNA methylation may indirectly impact H3 Lys9 trimethylation, consistent with recent results for dimethylated H3 Lys9 in *A. thaliana*[8].

To investigate possible global effects of *dim-5* and *dim-2* on dimethylated H3 Lys4 and trimethylated H3 Lys9 and to look for evidence of dimethylated H3 Lys9 in the genome, we carried out western-blot analyses of histones extracted from wild-type, *dim-5* or *dim-2* nuclei (Fig. 5). All the strains gave robust signals with the antibody against dimethylated H3 Lys4, comparable to that observed using control calf thymus histones. In contrast, no signal was detected in wild-type, *dim-5* or *dim-2* nuclei using the antibody against dimethylated H3 Lys9 under conditions that produced a strong signal with calf histones. These results extend our findings from ChIP experiments and suggest that *N. crassa* has little or no dimethylated H3 Lys9. Notably, trimethylated H3 Lys9 was detectable in bulk histones from the wild-type and *dim-2* strains. DNA methylation seems not to greatly impact global level of H3 Lys9 trimethylation in *N. crassa*. The *dim-5* mutation extinguished the trimethylated H3 Lys9 signal, suggesting that DIM-5 is responsible for most or all trimethylated H3 Lys9. The absence of detectable dimethylated H3 Lys9 in a *dim-5* strain suggests that DIM-5 generates trimethylated H3 Lys9 from unmodified H3 *in vivo*, as *in vitro*.

The results reported here, together with our finding that amino-acid substitutions of H3 Lys9 inhibit DNA methylation[10], implicate trimethylation of H3 Lys9 in the control of DNA methylation in *N. crassa*. In *D. melanogaster*, trimethylation of H3 Lys9 has recently been found to be associated with genes that are repressed by the Polycomb complex, which includes an H3 methyltransferase that can act on both Lys9 and Lys27 (ref. 13). Thus, one chromatin modification (for example, trimethylated H3 Lys9), alone or together with another modification, can be involved in more than one process, perhaps even in a single organism. Conversely, a given epigenetic process, such as DNA methylation, may be signaled in more than one way. Notably, evidence of dimethylated H3 Lys9 has been observed in methylated chromosomal regions of *A. thaliana*[8], raising the possibility that this mark can signal DNA methylation in some systems. Studies in mammals indicate that methylation of H3 Lys9 is an early step in X-chromosome inactivation, a process that also involves DNA methylation[7,16,27]. Curiously, the only protein currently known to specifically recognize methylated H3 Lys9, HP1 (refs. 28,29), has not been found to be associated with the inactive X chromosome[27]. Further research is needed to identify the signals leading to methylation of H3 Lys9 and the factors that read the trimethylated H3 Lys9 mark and trigger DNA methylation.

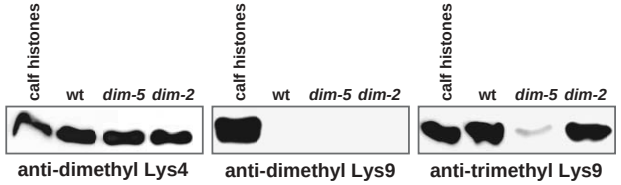

Fig. 5 Western-blot analysis of *N. crassa* histone H3 using antibodies against dimethylated H3 Lys4, against dimethylated H3 Lys9 or against trimethylated H3 Lys9. In each case, 5 μg calf thymus histones or 100 μg nuclear proteins from wild-type (74OR23-IVA; N150), *dim-5* (N2269) or *dim-2* (N2877) strains were fractionated by SDS–PAGE (18%) and analyzed using the indicated antibodies. Coomassie blue staining of a replica gel confirmed that intensity of bands corresponding to histones was approximately even between the wild-type, *dim-5* and *dim-2* samples. The blots for dimethylated and trimethylated H3 Lys9 were exposed roughly 5 times longer than that for dimethylated H3 Lys4.

Methods

Histone methyltransferase assay. We incubated 0.5 μg unmodified, dimethylated or trimethylated H3 Lys9 peptide (1–15; ARTKQTARKSTGGKA) for 1 h at 16 °C with 0.5 μg purified recombinant DIM-5 protein[15] and 1.1 μCi S-adenosyl-[methyl-^{3}H]-L-methionine (^{3}H AdoMet). We fractionated reaction products by SDS–PAGE (16.5%), fixed them with 10% glutaraldehyde for 15 min and fluorographed them to detect methylation as described[10]. We carried out DIM-5 assays with recombinant histone H3 N terminus as described[10].

Mass spectrometry analysis. We initiated DIM-5 reactions by adding 100 μM unmodified or dimethylated H3 Lys9 substrate (3–15; TKQTARKSTGGKA) to a 20-μl mixture of 50 mM glycine (pH 9.8), 10 mM dithiothreitol, 750 μM AdoMet and 2 μg DIM-5. After incubation at room temperature for the indicated times, we stopped the reactions by adding trifluoroacetic acid to 0.5%. We measured peptide masses by matrix-assisted laser desorption ionization time-of-flight on an Applied Biosystems Voyager System 4258 using α-cyano-4-hydroxycinnamic acid as matrix.

Characterization of antibodies. We spotted samples of unmodified, dimethylated or trimethylated H3 Lys9 peptides (1–15) onto a nitrocellulose membrane, fixed them with 10% glutaraldehyde for 15 min, stained them with Ponceau S and analyzed them by immunoblotting using antibodies against dimethylated[6] or trimethylated[18] H3 Lys9 according to the Immunoblot protocol (Upstate Biotechnology). We detected antibody–peptide complexes by horseradish peroxidase–conjugated goat antibody against rabbit IgG and chemiluminescence (PIERCE).

Western-blot analysis of *N. crassa* histones. We isolated *N. crassa* nuclei from wild-type strain 74OR23-IVA, *dim-5* strain N2269 (*dim-5 mat A*; ref. 10) and *dim-2* strain N1877 (*dim-2:hph his-3 mat a*; ref. 23) essentially as described[26] and lysed them in the buffer used for ChIP analysis[24]. We boiled nuclear lysates in SDS gel-loading buffer, clarified them by centrifugation, fractionated them by SDS–PAGE (18%), transferred them to polyvinylidene fluoride membranes in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11, containing 20% methanol at 70 V for 2 h and immunoblotted them as described above. Calf thymus histones were from Roche.

PCR primers. Sequences of pairs of PCR primers used to amplify fragments of *eta* (225 bp), *punt* (309 bp), *pcn* (470 bp) and *hH4* (425 bp) are available on request.

ChIP analysis. We carried out ChIP essentially as described previously[24] with modifications. Briefly, we germinated asexual spores (approximately 2×10^6 spores ml^{-1}) of the *N. crassa* wild-type strain N150 (74OR23-IVA) for 4.5 h in 50 ml of Vogel's minimal liquid medium supplemented with 1.5 % sucrose at 32 °C with shaking (200 r.p.m.). We grew cells of fission yeast strain SPG1335 to 10^7 cells ml^{-1} in 50 ml yeast extract adenine medium as described[3]. For chromatin fixation, we mixed both cultures, added paraformaldehyde to 2% and incubated them at 32 °C for 30 min with shaking (100 r.p.m.). After cell lysis, we sheared chromatin DNA to 0.5–0.8 kb and immunoprecipitated the soluble chromatin fraction using 2–8 μl of antibodies to dimethylated H3 Lys4, dimethylated H3 Lys9 or trimethylated H3 Lys9. We isolated DNA from immunoprecipitated chromatin, mock control chromatin or total chromatin and subjected it to PCR (95 °C, 30 s; 56 °C, 30 s; 72 °C, 1 min; 24 cycles for *N. crassa* chromatin regions; 30 cycles for *S. pombe* chromatin regions). PCR reactions (25 μl) included 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1 % Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dCTP, 0.2 mM dGTP, 2.5 μCi [α-^{32}P] dCTP, 0.5 μl sample DNA and 1.25 U *Taq* polymerase (Promega). We fractionated PCR products in 4% polyacrylamide gels, quantified band intensities using a STORM 860 Phosphorimager (Molecular Dynamics) and normalized the data based on results with total DNA. We found a linear relationship between the amount of input DNA and band intensities of the PCR products under the PCR condition (see Supplementary Fig. 3 online).

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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1. Strahl, B.D. & Allis, C.D. The language of covalent histone modifications. *Nature* **403**, 41–45 (2000).
2. Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D. & Felsenfeld, G. Correlation between histone lysine methylation and developmental changes at the chicken β-globin locus. *Science* **293**, 2453–2455 (2001).
3. Noma, K., Allis, C.D. & Grewal, S.I. Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* **293**, 1150–1155 (2001).
4. Santos-Rosa, H. *et al.* Active genes are tri-methylated at K4 of histone H3. *Nature* **419**, 407–411 (2002).
5. Rea, S. *et al.* Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**, 593–599 (2000).
6. Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. & Grewal, S.I. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science* **292**, 110–113 (2001).
7. Heard, E. *et al.* Methylation of histone H3 at Lys9 is an early mark on the X chromosome during X inactivation. *Cell* **107**, 727–738 (2001).
8. Johnson, L., Cao, X. & Jacobsen, S. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. *Curr. Biol.* **12**, 1360–1367 (2002).
9. Schotta, G. *et al.* Central role of *Drosophila* SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21**, 1121–1131 (2002).
10. Tamaru, H. & Selker, E.U. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414**, 277–283 (2001).
11. Jackson, J.P., Lindroth, A.M., Cao, X. & Jacobsen, S.E. Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416**, 556–560 (2002).
12. Tachibana, M., Sugimoto, K., Fukushima, T. & Shinkai, Y. Set domain-containing protein, G9a, is a novel lysine-preferring mammalian histone methyltransferase with hyperactivity and specific selectivity to lysines 9 and 27 of histone H3. *J. Biol. Chem.* **276**, 25309–25317 (2001).
13. Czermin, B. *et al. Drosophila* Enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal polycomb sites. *Cell* **111**, 185–196 (2002).
14. Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P. & Reinberg, D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. *Genes Dev.* **16**, 2893–2905 (2002).
15. Zhang, X. *et al.* Structure of the *Neurospora* SET domain protein DIM-5, a histone H3 lysine methyltransferase. *Cell* **111**, 117–127 (2002).
16. Boggs, B.A. *et al.* Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. *Nat. Genet.* **30**, 73–76 (2002).
17. Gendrel, A.V., Lippman, Z., Yordan, C., Colot, V. & Martienssen, R.A. Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene *DDM1*. *Science* **297**, 1871–1873 (2002).
18. Cowell, I.G. *et al.* Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. *Chromosoma* **111**, 22–36 (2002).
19. Selker, E.U. Premeiotic instability of repeated sequences in *Neurospora crassa*. *Annu. Rev. Genet.* **24**, 579–613 (1990).
20. Selker, E.U. *et al.* The methylated component of the *Neurospora crassa* genome. *Nature* (in the press).
21. Margolin, B.S. *et al.* A methylated *Neurospora* 5S rRNA pseudogene contains a transposable element inactivated by RIP. *Genetics* **149**, 1787–1797 (1998).
22. Hays, S.M., Swanson, J. & Selker, E.U. Identification and characterization of the genes encoding the core histones and histone variants of *Neurospora crassa*. *Genetics* **160**, 961–973 (2002).
23. Kouzminova, E.A. & Selker, E.U. *Dim-2* encodes a DNA-methyltransferase responsible for all known cytosine methylation in *Neurospora*. *EMBO J.* **20**, 4309–4323 (2001).
24. Nakayama, J., Klar, A.J. & Grewal, S.I. A chromodomain protein, Swi6, performs imprinting functions in fission yeast during mitosis and meiosis. *Cell* **101**, 307–317 (2000).
25. Singer, M.J., Marcotte, B.A. & Selker, E.U. DNA methylation associated with repeat-induced point mutation in *Neurospora crassa*. *Mol. Cell. Biol.* **15**, 5586–5597 (1995).
26. Rountree, M.R. & Selker, E.U. DNA methylation inhibits elongation but not initiation of transcription in *Neurospora crassa*. *Genes Dev.* **11**, 2383–2395 (1997).
27. Peters, A.H. *et al.* Histone H3 lysine 9 methylation is an epigenetic imprint of facultative heterochromatin. *Nat. Genet.* **30**, 77–80 (2001).
28. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**, 116–120 (2001).
29. Bannister, A.J. *et al.* Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**, 120–124 (2001).
30. Foss, H.M., Roberts, C J., Claeys, K.M. & Selker, E.U. Abnormal chromosome behavior in *Neurospora* mutants defective in DNA methylation. *Science* **262**, 1737–1741 (1993).