A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*

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DNA methylation is involved in epigenetic processes such as X-chromosome inactivation, imprinting and silencing of transposons. We have demonstrated previously that *dim-2* encodes a DNA methyltransferase that is responsible for all known cytosine methylation in *Neurospora crassa*. Here we report that another *Neurospora* gene, *dim-5*, is required for DNA methylation, as well as for normal growth and full fertility. We mapped *dim-5* and identified it by transformation with a candidate gene. The mutant has a nonsense mutation in a SET domain of a gene related to histone methyltransferases that are involved in heterochromatin formation in other organisms. Transformation of a wild-type strain with a segment of *dim-5* reactivated a silenced *hph* gene, apparently by 'quelling' of *dim-5*. We demonstrate that recombinant DIM-5 protein specifically methylates histone H3 and that replacement of lysine 9 in histone H3 with either a leucine or an arginine phenocopies the *dim-5* mutation. We conclude that DNA methylation depends on histone methylation.

Cytosine methylation is essential for the normal development of mammals and plants. Mutations in any of three known DNA methyltransferase (DMTase) genes of the mouse (*Dnmt1*, *Dnmt3a* and *Dnmt3b*) are lethal, either during embryogenesis or soon thereafter[1,2]. In humans, a syndrome characterized by immunodeficiency, centromere instability and facial anomalies results from mutations in the *DNMT3B* gene[3]. Reduction in methylation in the plant *Arabidopsis thaliana*, caused either by expression of an antisense construct against the DMTase MET1, or by mutation of a gene encoding a putative chromatin remodelling factor (*ddm1*), results in developmental abnormalities and partial female sterility[4].

In contrast to the situation in plants and animals, DNA methylation is not essential in the filamentous fungus *N. crassa*, thereby facilitating investigations of DNA methylation in this organism. As a step towards exploring the control and mechanism of cytosine methylation—processes that remain largely unknown in eukaryotes—we searched for methylation mutants in *Neurospora*. A screen of strains surviving a chemical mutagenesis yielded one mutant that was completely defective in methylation (*dim-2*), and another with an approximately 50% reduction in total DNA methylation (*dim-3*)[5]. The *dim-2* gene has recently been isolated and demonstrated to encode a DMTase that is responsible for both *de novo* and maintenance methylation at symmetrical and non-symmetrical sites[6]. Mutations in *dim-2* relieve silencing of methylated genes[7,8] but do not noticeably affect growth or development[6]. Mutants with defects in an unidentified *Neurospora* gene affecting methylation, *dim-1*, were found among 5-azacytidine-resistant derivatives of a DNA repair mutant, *mus-20* (ref. 9). The current study arose from an attempt to tag the *dim-1* gene by insertional mutagenesis in a *mus-20* strain.

We unexpectedly generated a mutation in a previously unknown gene required for DNA methylation, *dim-5*, and mapped the mutation to an 80-kilobase (kb) region. This region included a gene homologous to histone methyltransferases, which is required for heterochromatin formation in fission yeast, *Drosophila* and mammals. Transformation experiments confirmed that the candidate gene is *dim-5*, and biochemical tests on recombinant DIM-5 demonstrated that this protein methylates histone H3. The implication that histone methylation controls DNA methylation was supported by demonstrating that replacements of K9 in histone H3 cause loss of DNA methylation *in vivo*.

Isolation and genetic mapping of the *dim-5* mutation

We selected 150 strains that were resistant to 5-azacytidine from about 12,000 pRAL1 (*qa-2*$^+$) transformants of an *aro-9 qa-2 mus-20* strain (N2141), and tested them for methylation defects by Southern hybridization using probes for the ζ–η (ref. 5) and Ψ63 (ref. 10) methylated regions. One strain showed greatly reduced methylation in both regions. The methylation defect segregated in genetic crosses as expected of a normal mendelian allele; however, it did not co-segregate with either 5-azacytidine resistance or a pRAL1 insertion. Moreover, complementation tests between the new *dim* strain and previously identified mutants (*dim-1, dim-2* and *dim-3*) demonstrated that the mutant represents a new complementation group, which we designated *dim-5* (data not shown). Before detailed characterization, the *dim-5* mutation was purified from the mutagenized *mus-20* background by five backcrosses to a wild-type strain. Strains of *dim-5* showed slow, irregular growth (Fig. 1), unlike other methylation mutants, including *dim-2* (DMTase) null mutants (see Supplementary Information). In addition, homozygous *dim-5* crosses revealed a partial barren phenotype: few spores were produced, and most of those produced were inviable (data not shown).

About 1.5% of the cytosines in *N. crassa* DNA are methylated[5] and

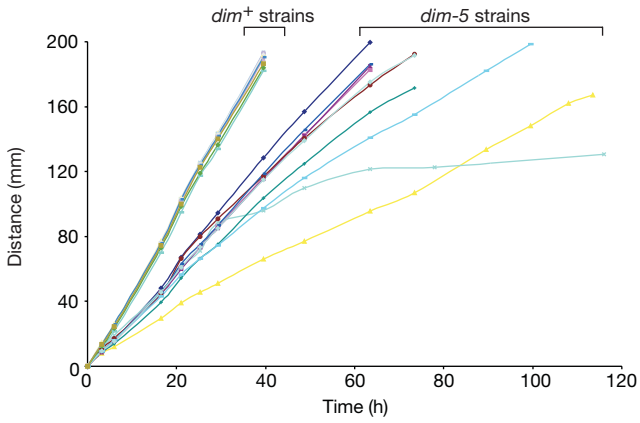

Figure 1 Growth deficiencies of *dim-5* strains. Rates of apical growth of ten wild-type (*dim*$^+$) and ten mutant (*dim-5*) progeny of N2140 (*dim-5 leu-2 pan-2* ***A***) × N185 (*trp-4* ***a***) were measured at 32 °C using 'race tubes' containing 1.5% sucrose Vogel's medium with pantothenate. The average rates and standard deviations of the wild-type and mutant strains were 4.9 ± 0.1 and 2.4 ± 0.7 mm h^{-1}, respectively. The rates of the ten wild-type strains were so similar that their plots are virtually superimposed.

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all known methylation is in relics of repeat-induced point mutation (RIP) and ribosomal DNA[11]. We therefore analysed four relics of RIP (ζ–η[11], Ψ63 (ref. 10), 1D21 and 9A20; T. Wolfe and E.U.S., unpublished data) and rDNA for methylation in *dim-5* strains. Southern hybridizations using the isoschizomers *Dpn*II and *Sau*3AI, which both recognize GATC but differ in that only *Sau*3AI is inhibited by cytosine methylation, revealed no methylation in *dim-5* strains at any of the five regions tested (Fig. 2). Inspection of total genomic DNA digested with *Dpn*II or *Sau*3AI and stained with ethidium bromide indicated that *dim-5*, like *dim-2*, eliminates all, or nearly all, DNA methylation (Fig. 2).

To identify the *dim-5* gene, we first mapped it by conventional genetics, scoring the mutation by Southern hybridization. Results of initial crosses localized the gene to linkage group IV. Analysis of three-point linkage data from a cross between a *dim-5 pan-1 pyr-2* strain (N2142) and a *trp-4* strain (N185) placed *dim-5* approximately two map units in a centromere-distal position of *trp-4* (see Supplementary Information). To determine whether *dim-5* is in the 2–4-map-unit region between *trp-4* and *leu-2*, or in a centromere-distal position of *leu-2*, we tested methylation of DNA from *trp-4*$^+$ *leu-2*$^+$ recombinant progeny from a cross of the *trp-4* strain with a *dim-5 leu-2* strain (N2140). Fifteen out of 42 recombinants were defective in methylation, establishing that *dim-5* is between *trp-4* and *leu-2*, a region in which no mutation had been mapped previously[12].

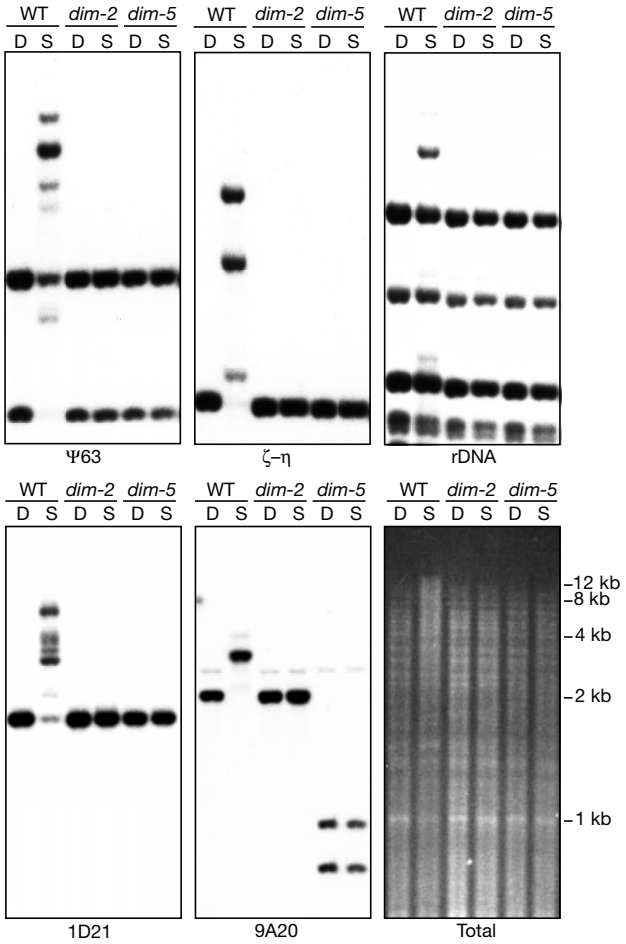

Figure 2 DNA methylation defect of *dim-5* strains. Genomic DNA of wild type (WT), a DNA methyltransferase mutant (*dim-2*) and the *dim-5* mutant were digested with *Dpn*II (D) or *Sau*3A1 (S), and analysed by gel electrophoresis and Southern hybridization using probes for the indicated five methylated chromosomal regions. The DNAs were stained with ethidium bromide (Total) to reveal the total digestion profiles generated with these isoschizomers. Blots were reprobed for unmethylated regions to confirm that digests were complete. The 9A20 region in the *dim-5* strain shows a restriction-fragment length polymorphism relative to the wild-type and *dim-2* strains.

Identification of the *dim-5* open reading frame

As a step towards identifying the *dim-5* gene, we searched *N. crassa* genomic sequence data (Neurospora Sequencing Project; http://www-genome.wi.mit.edu) for *leu-2* and *trp-4* on the basis of their expected homology to *Saccharomyces cerevisiae LEU1* and *TRP4*, respectively[12]. Candidates for both genes were found separated by about 80 kb, consistent with the genetic distance between these genes. We examined the interval between the putative *leu-2* and *trp-4* genes for *dim-5* candidates (Fig. 3a). One of 15 candidates identified using BLASTx is predicted to encode a protein that is related to chromatin-associated proteins involved in gene silencing in fission yeast and fruit flies, namely *Schizosaccharomyces pombe* Clr4 (ref. 13) and *Drosophila melanogaster* Su(var)3-9 (ref. 14). Mutations in *clr4* derepress silent mating-type genes and genes inserted in other repressive domains and cause aberrant chromosome behaviour[15], whereas mutations in *su(var)3-9* suppress the gene silencing phenomenon known as position effect variegation in *Drosophila*[14]. To test the possibility that the related *Neurospora* gene is required for DNA methylation in *Neurospora*, we tested fragments from the *leu-2*/*trp-4* region for complementation of the *dim-5* mutation. Because co-transformation is highly efficient in *Neurospora*, we transformed a *dim-5* strain with a mixture of a plasmid (pBT6) conferring resistance to benomyl (Bml^r) plus a test fragment, and then assayed random Bml^r transformants for *de novo* methylation of Ψ63. Southern blot analysis of 26 Bml^r transformants generated with a 4.3-kb DNA fragment containing the *clr4*/*su(var)3-9* homologue, plus a putative 3-hydroxyisobutyrate dehydrogenase (*hibD*) gene, showed that all 24 transformants that incorporated the non-selected fragment displayed substantial *de novo* DNA methylation at the Ψ63 and ζ–η regions and in the genome overall (see Supplementary Information). Equivalent results were obtained with smaller restriction fragments including the *clr4*/*su(var)3-9*-related gene alone (Fig. 3a, b). We therefore tentatively concluded that this open reading frame (ORF) is *dim-5*.

Derepression of a silenced transgene by quelling *dim-5*

It remained formally possible that ectopic insertions of the *Neurospora clr4*/*su(var)3-9* homologue suppressed the *dim-5* mutation owing to a dosage effect, but was not itself *dim-5*. We took advantage of quelling—a post-transcriptional gene silencing mechanism of *Neurospora* that does not depend on DNA methylation[16]—to carry out an independent test of the *clr4*/*su(var)3-9* homologue. In particular, we tested whether introduction of fragments of the presumptive *dim-5* coding region into a Dim$^+$ *Neurospora* strain (N644) carrying a methylated, silent transgene (*hph*), would silence the wild-type *dim-5* gene and thereby activate the transgene (Fig. 4a). We co-transformed N644 with a mixture of pBT6 and an *Eco*RV digest of a PCR fragment containing the entire presumptive *dim-5* ORF, and tested Bml^r transformants for resistance to hygromycin (Hyg^r), which should result if the *hph* gene were demethylated. Five out of twelve Bml^r transformants that were generated with the DNA mixture showed strong resistance to hygromycin and several others showed partial resistance (Fig. 4b). In contrast, none of the control transformants generated with pBT6 alone were resistant to hygromycin. To test directly for loss of DNA methylation, we isolated DNA from these strains grown under selection for benomyl, but not hygromycin, and assayed methylation at the Ψ63 region (Fig. 4c). Substantial hypomethylation was observed and was correlated with activation of the silenced *hph* gene. These results therefore confirmed our tentative identification of *dim-5* and demonstrated that this gene is subject to quelling.

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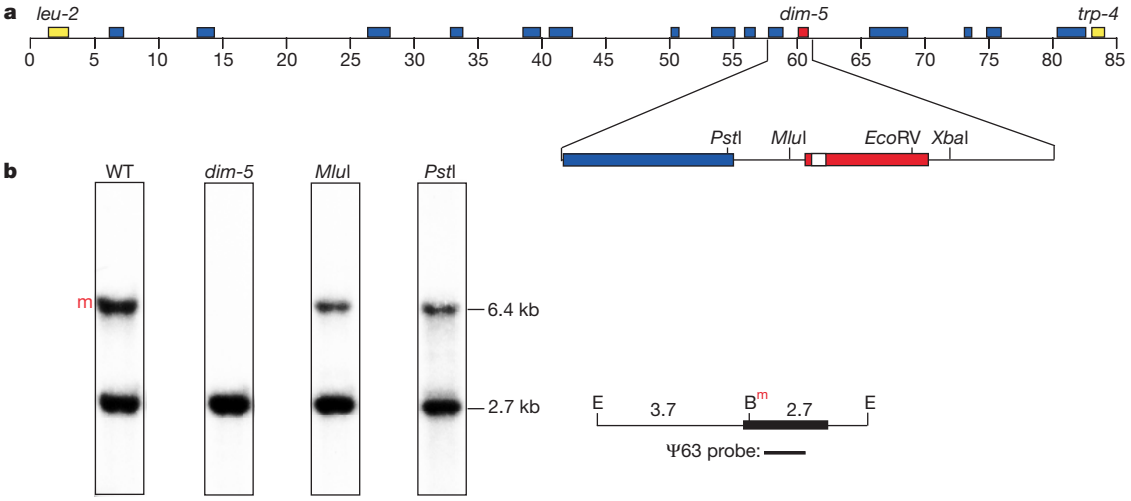

Figure 3 Identification of *dim-5* by genetic mapping and complementation. **a**, Map of genes revealed by BLASTx in *leu-2*/*trp-4* interval of contig 1.118 (assembly version 1; http://www-genome.wi. mit.edu). Regions of marked similarity (alignment scores >80) to genes in NCBI database are indicated (rectangles). A segment that was amplified to test two *dim-5* candidates (*hibD* gene, blue; homologue of *S. pombe clr4*, red with white intron) is shown expanded. **b**, Complementation of *dim-5* mutation. *dim-5* strain N2145 was co-transformed with pBT6 and a 2.0-kb *PstI*/*XbaI* or 1.4-kb *MluI*/*XbaI* fragment. Genomic DNA from representative Bml^r transformants was analysed by Southern hybridization for DNA methylation in the Ψ63 region using *Eco*RI (E) and *Bam*HI (B). Methylation of the *Bam*HI site[10] gives a 6.4-kb fragment, as shown (red m). Results for representative transformants are shown with positive (WT) and negative (*dim-5*) controls.

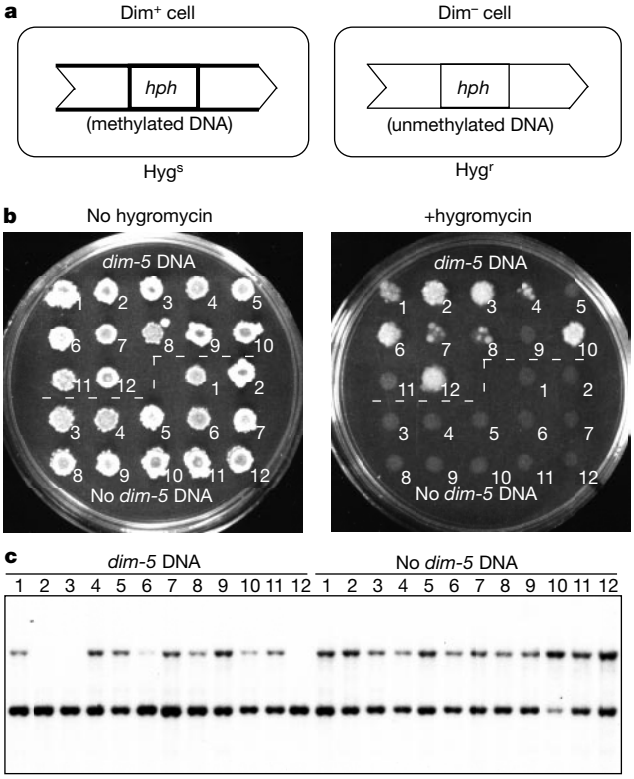

Figure 4 Quelling of *dim-5* relieves silencing of *hph* in N644. **a**, Sketch of methylated (Hyg^s) or unmethylated (Hyg^r) *hph* gene, flanked by methylation-inducing DNA segments that had been subjected to RIP[49]. **b**, Conidia of 24 random transformants of strain N644, generated with pBT6 alone or with pBT6 plus a PCR fragment digested with *Eco*RV containing *dim-5* (see Methods), were spot-tested for growth in the absence or presence of hygromycin. **c**, Effect of *dim-5* gene fragments on methylation at Ψ63. The transformants shown in **b** were analysed for methylation as in Fig. 3.

The *dim-5* mutant has a nonsense mutation in the SET domain

As a final test of whether we had correctly identified the *dim-5* gene, we amplified (using PCR) and sequenced the ORF from our mutant and from its wild-type parental strain. We found a single C to G mutation in the serine codon (TCA) at amino acid position 216 of the predicted 318-amino-acid polypeptide. The mutation generated a stop codon in the middle of a distinctive ~130-amino-acid sequence motif called the SET domain (Fig. 5). We conclude that DIM-5 is a SET domain protein, which is homologous to genes required for heterochromatin formation.

The SET domain was initially identified as a region of apparent homology in three nuclear proteins of *Drosophila*: Su(var)3-9, the Polycomb group protein E(Z) and Trithorax group protein TRX[14]. Greater than 200 genes with SET domains are now known[17]. Like *clr4*, *su(var)3-9*, *SUV39H1*, *Suv39h1* and *Suv39h2*, *dim-5* includes cysteine-rich sequences that flank a SET domain (Fig. 5). Noting that some SET proteins are protein methyltransferases, the possibility that chromatin-associated SET domain proteins are histone methyltransferases (HMTases) was examined[18]. Although not detected with all the chromatin-associated SET proteins, *S. pombe* Clr4 (refs 18, 19) and the closely related proteins from humans (SUV39H1 (ref. 18) and mouse (Suv39h1 (ref. 18) and Suv392h2 (ref. 20) did show HMTase activity. Analyses of variants generated *in vitro* indicated that both the SET domain and the associated cysteine-rich sequences are required for HMTase activity[18,19].

dim-5 encodes a histone H3 methyltransferase

We directly investigated the possibility that DIM-5, like Clr4 and Suv39h2, is a histone methyltransferase. We built an inducible glutathione *S*-transferase (GST) fusion construct containing nearly all of the DIM-5 coding region, similar to constructs made to assay HMTase activity of Clr4 and Suv39h2 (Fig. 5b). Recombinant DIM-5 fusion protein was purified from *Escherichia coli*, provided with *S*-adenosyl-[methyl-3H]-L-methionine as a potential methyl-group donor, and incubated with a natural mixture of histones from calf thymus. The proteins were then fractionated by SDS–polyacrylamide gel electrophoresis (PAGE) and assayed for incorporation of methyl groups by fluorography and scintillation counting of gel slices. We detected significant incorporation of

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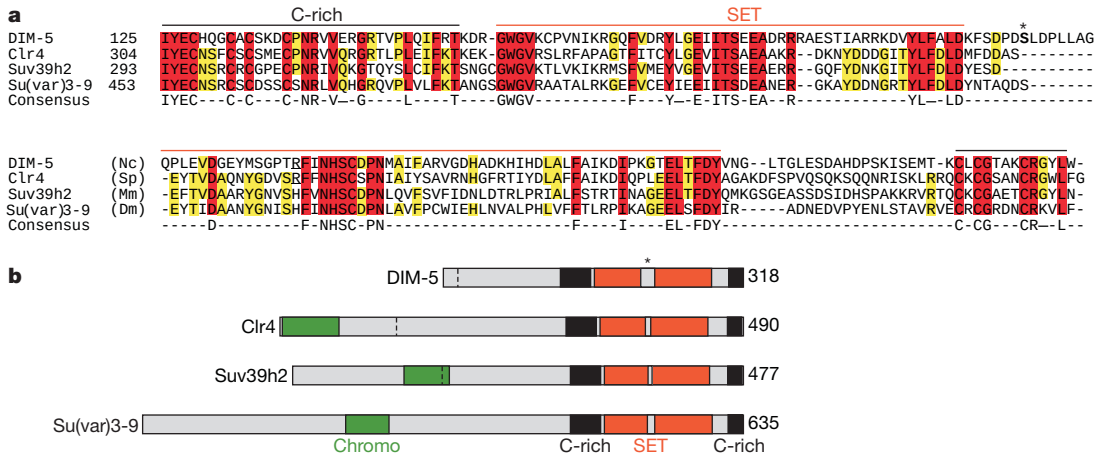

Figure 5 DIM-5 is a SET-domain protein. **a**, Amino acid alignment of conserved regions of *N. crassa* DIM-5 (accession number AF419248), *S. pombe* Clr4 (accession number O60016), *Mus musculus* Suv39h2 (accession number NM 022724.1) and *D. melanogaster* Su(var)3-9 (accession number S47004) generated with CLUSTALW (http://workbench.sdsc.edu). Amino acid coordinates and species abbreviations are shown at the left. Nc, *N. crassa*; Sp, *S. pombe*; Mm, *M. musculus*; Dm, *D. melanogaster*. The position of a nonsense mutation in allele *dim-5* HT1 is indicated with an asterisk. Amino acid residues conserved among all four proteins are highlighted in red and indicated in 'consensus'; residues conserved in three of the four proteins are highlighted in yellow; dashes indicate gaps in the alignment. The black and orange lines mark the extent of the cysteine-rich (C-rich) and SET domains, respectively. **b**, Protein domain organization of DIM-5 and related proteins aligned at their carboxy termini with predicted number of amino acids and locations of chromo (green) SET (orange) and cysteine-rich (black) domains indicated. The amino-terminal endpoints of recombinant proteins made in this study or previously[18] are indicated by vertical dashed lines.

labelled methyl groups into histones, indicating that DIM-5 is an HMTase. Moreover, histone H3 was the acceptor of nearly all the methylation, as found with Clr4 and Suv39h2 (Fig. 6). Much weaker incorporation of label was detected in a protein tentatively identified as a histone H1 (data not shown).

Lysine 9 of histone H3 gene is involved in DNA methylation

Our demonstration that DIM-5 has HMTase activity suggested that methylation of histone H3 is necessary for DNA methylation in *Neurospora*. It remained possible, however, that the HMTase activity was not relevant to its role in DNA methylation. We therefore sought *in vivo* evidence for involvement of histone H3 in DNA methylation. The Clr4 and Suv39h2 HMTases are specific for K9 of H3 (refs 18, 19), a residue that can be either methylated or acetylated[17] (Fig. 7a). We reasoned that if methylation of K9 of histone H3 were required for DNA methylation in *Neurospora*, then replacement of this residue with an amino acid that is not subject to this modification should interfere with DNA methylation. We therefore mutated the H3 gene (*hH3*) *in vitro*, replacing the lysine codon with codons for leucine or arginine, and introduced the modified genes into strain N644 by co-transformation. Leucine and arginine were chosen for the following reasons: (1) they are structurally similar to lysine; (2) the neutral amino acid leucine can be regarded as a mimic of an acetylated lysine; (3) the positively charged amino acid arginine can be regarded as a mimic of an unacetylated lysine; and (4) leucine is known not to be a substrate for methylation of recombinant Suv39h1 HMTase[18].

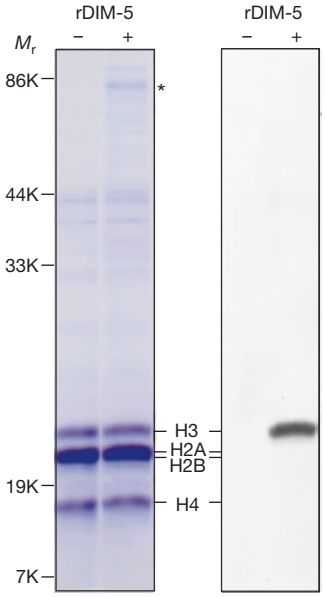

Figure 6 Histone methyltransferase activity of recombinant DIM-5 protein (rDIM-5). Purified histones (~20 μg; Boehringer Mannheim) were incubated for 6 h at 20 °C with or without purified GST–DIM-5 fusion protein (GST–DIM-5; ~1 μg) and 2.75 μCi *S*-adenosyl-[methyl-3H]-L-methionine, as methyl donor. Reaction products were fractionated by PAGE (16.5%), stained with Coomassie blue (left) and then fluorographed (right) to detect methylation. The positions of selected size standards, intact recombinant protein (asterisk) and core histones are indicated.

Neurospora crassa has only a single copy of the *hH3* gene[21] but gene replacement by homologous recombination is inefficient in *Neurospora*. Furthermore, replacement of the wild-type *hH3* with the mutated versions might be lethal. It seemed possible, however, that the mutations would prove dominant or semi-dominant. We therefore took advantage of the methylated *hph* allele of N644 to test for loss of DNA methylation in random transformants generated with the mutated *hH3* constructs. Transformants generated with mutant or wild-type *hH3* genes together with the co-transformation marker, *Bml*, were selected *en masse* on benomyl medium and then tested for expression of *hph*. About 500 asexual spores from each pool, representing roughly 30 Bml^r transformants, were spread on hygromycin plates (Fig. 7b). Hyg^r colonies were obtained with both mutants, but not with the wild-type control, in two independent experiments. Southern hybridization analyses of DNA isolated from representative Hyg^r transformants demonstrated that the strains had markedly reduced DNA methylation at Ψ63, and confirmed that they contained modified *hH3* alleles (Fig. 7c). The Hyg^r transformants in each experiment contained a single ectopic copy of the mutant allele, which is unusual for *Neurospora*. Perhaps

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additional copies of the mutant *hH3* genes were toxic, either directly or because they caused quelling, thereby reducing H3 levels beyond the point that the cells could survive. Direct DNA sequencing of *hH3* PCR products confirmed that the strains contained both the wild-type and mutant sequences (Fig. 7c). These results strongly support the inference from our other results that methylation of histone H3 is critical for DNA methylation.

Does RIP generate heterochromatin?

The densely staining 'constitutive heterochromatin', which is frequently found near centromeres and telomeres in eukaryotes, provides an example of how DNA sequences can direct the formation of specialized forms of chromatin. Constitutive heterochromatin is typically rich in moderately repeated sequences (such as transposons) and highly repeated sequences (such as satellite DNA) and displays a number of other identifying characteristics. It remains condensed after mitosis, replicates late in S phase, shows low levels of genetic recombination, contains special forms of histones, and, in organisms with DNA methylation such as mammals and plants, it is hypermethylated[22]. Classical studies in *Drosophila* have demonstrated that heterochromatin is a repressive environment for most genes; rearrangements that move heterochromatic regions typically cause silencing of nearby genes in some fraction of the cells. Isolation of suppressors of the resulting variegation identified the heterochromatin protein HP1 (Su(var)3-5)[23], an HMTase (Su(var)3-9)[14,18] and an *S*-adenosylmethionine synthetase (Su(z)5)[24]. The very much smaller heterochromatic regions of *S. pombe* depend on a similar set of silencing genes, including the chromo domain genes *swi6* and *clr4*, which encode a HP1-like protein and a histone H3 MTase, respectively[19]. The chromo domain of HP1 has recently been shown to recognize methylated K9 of histone H3 (refs 25, 26).

Our finding that DNA methylation in *Neurospora* relies on histone H3 K9 raises the possibility that sequences mutated by RIP, which constitute the bulk of methylated sequences of this organism and are found concentrated in centromeric DNA[27], serve to nucleate heterochromatin. RIP detects duplicated sequences, such as transposons[10,27], during the sexual phase of the *Neurospora* life cycle and peppers them with G·C to A·T mutations[11]. Like some satellite sequences, products of RIP are bound by proteins that show limited sequence specificity (H.T., G. Kothe and E.U.S., unpublished observations). These proteins may be responsible for recruiting heterochromatin proteins—perhaps the DIM-5 HMTase. Thus, relics of RIP may underlie heterochromatin in *Neurospora* and serve the cell for centromere function. Our observation that *dim-5* strains grow irregularly and are nearly sterile in homozygous crosses, unlike other known DNA methylation mutants, indicates that DIM-5 is involved in process(es) besides DNA methylation such as in heterochromatin formation.

Histones as signal transducers for DNA methylation

The control of DNA methylation has remained unclear despite decades of intensive investigations in mammals, plants and fungi. Although prokaryotic and eukaryotic DMTases show marked structural similarities, prokaryotes offer an inappropriate model for DNA methylation in eukaryotes. Bacterial DMTases require nothing more than DNA and a methyl-group donor for proper function, and are sequence specific. In contrast, eukaryotic DMTases have substantial non-catalytic domains that reflect interactions with other proteins[28], and show little sequence specificity[29]. We suggest that eukaryotic DMTases evolved to take their cues primarily from chromatin. A common view is that histones act as obstacles to proteins that need access to the DNA. The discovery of chromatin-remodelling factors that can move nucleosomes[30], and of a putative chromatin-remodelling factor involved in DNA methylation[31], fit this view. Our discovery that DNA methylation depends on histone methylation in *Neurospora* suggests an alternative possibility, however. Proteins that require access to DNA, such as DMTases, may actually depend on histones as cofactors. According to this view, chromatin-remodelling factors may assist histone modification enzymes to reach their targets and/or to facilitate exchange of nucleosomes with different modification states.

Histones are well suited to integrate information relevant to whether DNA in a particular region should be methylated. In addition to their proximity to DNA, histones are subject to a variety

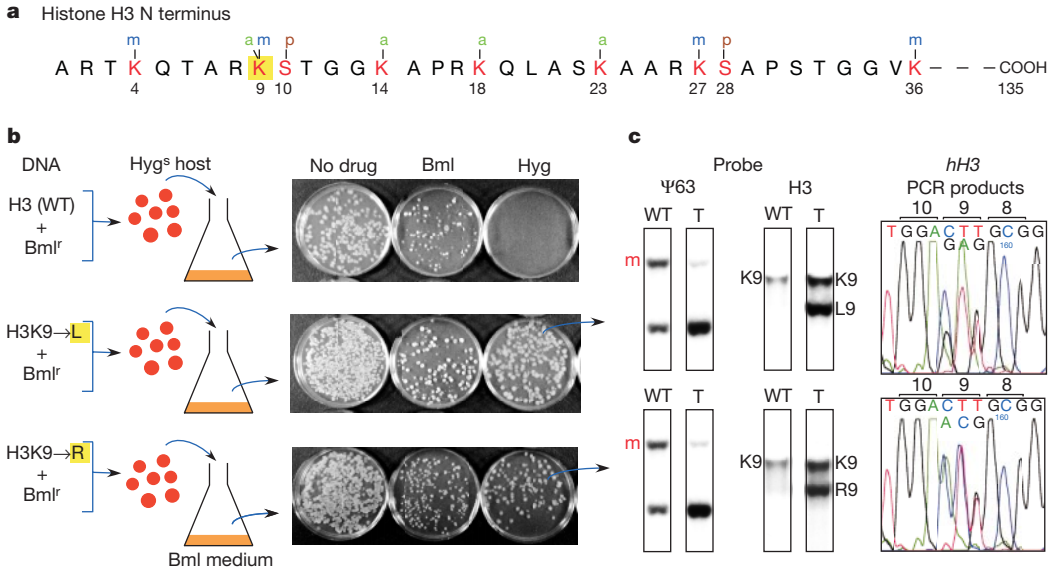

Figure 7 Reactivation of *hph* and loss of DNA methylation induced by transformation of a *dim*$^+$ strain with mutant alleles of histone H3 gene (*hH3*). **a**, Sequence of the N-terminal segment of *Neurospora* histone H3 with residues presumed to be subject to methylation (m), acetylation (a) or phosphorylation (p) indicated in red and the residue implicated in silencing highlighted in yellow. **b**, Transformation experiment. Strain N644 (see Fig. 4) was co-transformed with pBT6 and wild-type or mutant versions of *hH3*, and Bml^r transformants were selected *en masse* on benomyl and tested for drug resistance. **c**, Southern analysis and sequencing of DNA from Hyg^r transformants. DNA of representative transformants (T) and a wild-type (WT) control grown non-selectively was analysed with *Eco*RI and *Bam*HI for methylation (m) at Ψ63 as in Fig. 3, and for ectopic alleles of *hH3*. Direct sequencing of *hH3* PCR products confirmed the presence of both the wild-type and mutant alleles in representative strains (sequencing chromatograms).

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of post-translational modifications (phosphorylation, methylation, acetylation, ubiquitination and ADP-ribosylation) that can have informational roles in the cell[32]. Acetylation, currently the best understood modification, is controlled by histone acetylases (HATs) and histone deacetylases (HDACs), which typically act as transcriptional coactivators and corepressors, respectively. Mechanistic connections are emerging among DNA methylation, histone deacetylation and histone methylation. Mammalian methyl-DNA binding proteins and DMTases associate with HDACs, and the histone deacetylase inhibitor trichostatin A (TSA) causes selective loss of DNA methylation in *Neurospora*, suggesting that histone acetylation can influence DNA methylation[33,34]. Moreover, in *S. pombe*, TSA treatment or mutation of HDAC genes causes mislocalization of Swi6 and other defects characteristic of disruption of the *clr4* HMTase[19,35,36], perhaps because methylation of K9 of histone H3 is inhibited by acetylation of K9 or 14 (refs 18, 19). Phosphorylation of S10 also strongly inhibits methylation of K9 (ref. 18), providing another illustration of how histones can integrate information from multiple inputs and act as signal transducers. There are potentially a huge number of combinations of variously modified histones, and the network that connects modifications of DNA and chromatin proteins may be complex.

De novo and maintenance methylation of DNA in eukaryotes

A defining feature of epigenetic states is that they promote their own propagation. Thus, active chromosomal regions are rarely silenced and silenced regions are rarely activated. It was recognized previously that the symmetry of methylated sites (5′-CpG/GpC-5′) in mammalian DNA would support a simple mechanism to propagate methylation patterns; all that was required was a DMTase specific for hemi-methylated sites[37]. The 'maintenance methylase' model was supported by evidence that methylation states are indeed propagated, and by the discovery of DMTases that prefer hemi-methylated substrates. Nevertheless, the classic maintenance model does not account for some observations, such as heterogeneous methylation in cell clones, spreading of methylation and stable propagation of methylation at non-symmetrical sites, as observed in *Neurospora* and other eukaryotes[38–40,50]. Now that we know that histone modifications can impact both *de novo* and maintenance DNA methylation, we should consider the possibility that propagation of DNA methylation patterns in eukaryotes depends on feedback loops between modifications of chromatin proteins and DNA. Of note, histones H3 and H4 remain tightly bound to DNA *in vivo*, unlike histones H2A and H2B[41]. This is consistent with the idea that these histones are involved in the propagation of epigenetic states.

In *Drosophila* and *S. pombe*, interplay between heterochromatin proteins propagates epigenetic states without relying on DNA methylation[42–44]. If the chromo domains of Clr4 and Su(var)3-9 recognize methylated K9 of histone H3, as with HP1 (refs 25, 26), this might lead to preferential methylation of histones in previously methylated regions, which would propagate silencing. The absence of the chromo domain from the DIM-5 HMTase, and from the recently described G9a HMTase[45], is interesting. Perhaps DNA methylation and associated factors (for example, DMTases and methyl-DNA binding proteins) substitute for this potential self-reinforcing system. DMTases containing a chromo domain have been identified in plants[46], suggesting that some DMTases may take cues directly from histones. A search of public databases with DIM-5 revealed a number of potential HMTases that may be involved in DNA methylation. Future work will reveal the detailed relationship between methylation of histone H3 and DNA in *Neurospora*, and will show whether this 'double methylation' silencing system is widely used in eukaryotes. □

Methods

PCR primers are listed in Supplementary Information.

Analysis of DNA methylation

Genomic DNA was isolated from liquid cultures grown for 2 days at 32 °C, and analysed for DNA methylation by Southern hybridization as previously described[5]. The probe for the Ψ63 region was a 0.9-kb *Ban*II/*Eco*O109 fragment isolated from pPG22 (ref. 10). The 0.8-kb *Bam*HI fragment was used to probe for the ζ–η region, and a 9.2-kb *Kpn*I fragment representing one repeat unit of the rDNA was used was used to probe for rDNA. The 1D21 and 9a20 probes were generated by PCR from the wild-type strain 74-OR23-IVA. Strains used for the methylation analysis shown in Fig. 2 were: 74-OR23-IVA (wild type; all blots), *dim-2* strains N1275 (ref. 5) (*dim-2 arg-10 mat* ***A***; Ψ63, ζ–η and rDNA blots) and N1877 (ref. 6) (*dim-2*::*hph his-3 mat* ***a***; 1D21 and 9A20 blots), and *dim-5* strains N2144 (*dim-5*; Ψ63, ζ–η and rDNA blots), N2145 (*dim-5 leu-2 pan-2* ***a***; 1D21 blot) and N2140 (*dim-5 leu-2 pan-2* ***A***; 9A20 blot).

Complementation tests and quelling experiments

Complementation of *dim-5* was accomplished with either a 4.3-kb *hibD*/*dim-5* PCR fragment amplified from wild-type strain 74-OR8-1a with *Pfu* DNA polymerase (Promega), or restriction fragments of this PCR fragment (Fig. 3a). Strain N2145 was co-transformed by electroporation[47] using 100 μg pBT6, which confers resistance to benomyl (ref. 48), and 300 μg test DNA.

For quelling, a PCR fragment containing the presumed *dim-5* ORF was generated, cut within the SET domain of the gene with *Eco*RV, and introduced into strain N644 (ref. 49) (*dim-5*$^{+}$ *am*^{RIP}/*hph*/*am*^{RIP}*am*^{132} *inl* ***a***) by co-transformation with pBT6. Approximately 10^4 conidia of representative Bml^r transformants were spot-tested on Vogel's sorbose medium in the presence or absence of hygromycin (200 μg ml^{-1}; Calbiochem). Genomic DNA was isolated from liquid cultures grown for 2 days at 32 °C, and analysed for DNA methylation[5].

Identification of mutation in *dim-5* allele HT1

To identify the mutation in *dim-5* allele HT1 of strain N2140, pooled PCR products from six independent reactions produced with *Pfu* DNA polymerase and the *dim-5* ORF primers were gel-purified and sequenced directly on both strands. The wild-type allele (Fungal Genetics Stock Center (FGSC) 988) was isolated and sequenced in the same way (GenBank accession code AF419248).

Generation and purification of GST–DIM-5 fusion protein

A segment of the *dim-5* ORF, including amino acid residues 19–318, was amplified from *Neurospora* wild-type strain 74-OR8-1a (FGSC 988) with *Pfu* DNA polymerase and GST–DIM-5 primers. The PCR product was digested with *Bam*HI and *Eco*RI, gel-purified and cloned into the GST fusion expression vector pGEX-5X-3 (Pharmacia) using *E. coli* strain DH5αF′. Recombinant protein was prepared from an 800-ml culture of *E. coli* cells grown 3 h at 37 °C in LB medium with ampicillin (400 μg ml^{-1}), shifted to 30 °C for 40 min, induced with IPTG (0.1 mM) and collected 1 h later. Cells were lysed by sonication on ice in 8 ml of RIPA buffer (20 mM Tris (pH 7.5), 500 mM NaCl, 5 mM EDTA, 1% IGEPAL CA-630 (Sigma), 0.5% deoxycholate)[18] with 5 mg ml^{-1} lysozyme and a proteinase inhibitor cocktail (Complete; Boehringer Mannheim). The extract was clarified by centrifugation and the soluble proteins were incubated at 4 °C for 15 min with 700 μl glutathione-agarose (Sigma) equilibrated in RIPA. GST/DIM-5 protein was purified by washing three times with 30 ml RIPA buffer, followed by elution from glutathione-agarose using 75 mM HEPES (pH 7.9), 150 mM NaCl, 5 mM dithiothreitol and 10 mM reduced glutathione (Sigma). The eluate was concentrated with a Centricon-30 filter (Millipore).

Histone methyltransferase assay

HMTase assays were carried out on a natural mixture of calf thymus histones as described[18], except that the reaction was carried out at 20 °C for 6 h with 2.75 μCi *S*-adenosyl-[methyl-3H]-L-methionine (0.55 mCi ml^{-1}; NEN). Products were fractionated on SDS–PAGE (16.5%; 29:1) gels and fluorographed (4–12 h) using ENTENSIFY (DuPont).

In vitro mutagenesis and *in vivo* analysis of histone H3 variants

K9 of *N. crassa* histone H3 (ref. 21) was changed to leucine and arginine using the PCR-based QuickChange site-directed mutagenesis protocol (Stratagene) with a 4.9-kb plasmid carrying the wild-type H3 gene (*hH3*) and 1,161 bp of 5′-flanking sequences (pSH12; S. Hays and E.U.S., unpublished data) as template. Primer pairs H3L9 and H3R9 were used to generate CTC and CGT codons in place of the AAG codon, respectively. The resulting plasmids (pSH12L9 and pSH12R9, respectively) and the wild-type control were linearized using *Xba*I and co-transformed into *N. crassa* strain N644 along with pBT6 that was linearized with *Hin*dIII. Approximately 1,000 conidia from transformants grown *en masse* on solidified Vogel's sucrose medium in flasks were plated on media containing no drug, benomyl (0.5 μg ml^{-1}) or hygromycin (200 μg ml^{-1}). Random Hyg^r transformants, which were obtained with pSH12L9 and pSH12R9 but not with pSH12 (Fig. 7), were grown in liquid medium in the absence of hygromycin to isolate DNA. The presence of ectopic *hH3* sequences with the expected mutations was verified by Southern hybridization and direct sequencing of PCR products generated using H3-ORF primers.

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