DOI: 10.1534/genetics.110.123315

H2B- and H3-Specific Histone Deacetylases Are Required for DNA Methylation in *Neurospora crassa*

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Manuscript received August 12, 2010
Accepted for publication September 18, 2010

ABSTRACT

Neurospora crassa utilizes DNA methylation to inhibit transcription of heterochromatin. DNA methylation is controlled by the histone methyltransferase DIM-5, which trimethylates histone H3 lysine 9, leading to recruitment of the DNA methyltransferase DIM-2. Previous work demonstrated that the histone deacetylase (HDAC) inhibitor trichostatin A caused a reduction in DNA methylation, suggesting involvement of histone deacetylation in DNA methylation. We therefore created mutants of each of the four classical *N. crassa* HDAC genes and tested their effect on histone acetylation levels and DNA methylation. Global increases in H3 and H4 acetylation levels were observed in both the *hda-3* and the *hda-4* mutants. Mutation of two of the genes, *hda-1* and *hda-3*, caused partial loss of DNA methylation. The site-specific loss of DNA methylation in *hda-1* correlated with loss of H3 lysine 9 trimethylation and increased H3 acetylation. In addition, an increase in H2B acetylation was observed by two-dimensional gel electrophoresis of histones of the *hda-1* mutant. We found a similar increase in the *Schizosaccharomyces pombe* Clr3 mutant, suggesting that this HDAC has a previously unrecognized substrate and raising the possibility that the acetylation state of H2B may play a role in the regulation of DNA methylation and heterochromatin formation.

METHYLATION of selected cytosines in DNA is common in animals, plants, and fungi and serves in both genome defense and gene regulation (Selker 2004; Zilberman 2008). Despite recent progress in understanding the mechanisms of DNA methylation, its regulation remains largely undefined. For example, the extent to which various histone modifications affect DNA methylation has not been established. The filamentous fungus *Neurospora crassa* provides a particularly favorable model system to investigate such issues. DNA methylation in *N. crassa* is governed by DIM-5, a histone methyltransferase (HMTase) that trimethylates H3 lysine 9 (H3K9) (Tamaru and Selker 2001; Tamaru *et al.* 2003). Methylated H3K9 is bound by heterochromatin protein-1 (HP1) (Freitag *et al.* 2004a), which directly recruits the DNA methyltransferase (DMTase) DIM-2 (Honda and Selker 2008). Phosphorylation of H3 serine 10 (H3S10) interferes with methylation of H3K9 by DIM-5 (Adhvaryu and Selker 2008). This study was motivated by an early indication that the acetylation state of one or more histones bears on DNA methylation. In particular, we noted that the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) inhibited some DNA methylation in *N. crassa* (Selker 1998). TSA can also influence DNA methylation in plant and animal systems (Chen *et al.* 1998; Cameron *et al.* 1999; Ou *et al.* 2007). Because methylation of H3K9 is required for DNA methylation, a simple hypothesis was that removal of an acetyl group from this residue is required to generate a substrate for the H3K9 MTase, DIM-5. Moreover, it seemed possible that acetylation of other residues, such as H3K14, might indirectly influence DNA methylation, for example, by interference with the binding of HP1 (Mateescu *et al.* 2004). Other possibilities exist of course.

Histone deacetylases have been shown to play prominent roles in the regulation of gene expression (Cunliffe 2008; Hollender and Liu 2008; McDonel *et al.* 2009). Extensive work in yeasts, which lack DNA methylation, has linked particular histone acetylation to cases of both gene activation and repression (Robyr *et al.* 2002; Wiren *et al.* 2005; Sinha *et al.* 2006). A phylogenetic tree based on a single alignment of protein sequences of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* HDACs and their *N. crassa* homologs is shown in Figure 1. Actively

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Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.123315/DC1.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. HQ625364–HQ625365.

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transcribed chromatin regions in *S. cerevisiae* are enriched for H3 acetylation on K9, K18, and K27 and lack acetylation on H4 K16 and H2B K11 and -K16 (Kurdistani *et al.* 2004). Rpd3 and Hda1 are responsible for deacetylation of the promoters of distinct sets of genes (Robyr *et al.* 2002). Rpd3 is a global repressor, and mutants lacking this enzyme show increased acetylation of H4 K5 and -K12 and H3 K18 at derepressed genes (Robyr *et al.* 2002). Hda1 deacetylates subtelomeric regions as well as the promoters of a large set of genes, most of which do not depend on Rpd3.

How HDACs would be involved in DNA methylation is not obvious, but similarities between known components of the heterochomatin/DNA methylation machinery of Neurospora and the heterochromatin machinery of *S. pombe* (which lacks DNA methylation) suggest that information from *S. pombe* may provide clues. As in Neurospora, heterochromatin formation in *S. pombe* requires an H3 K9 HMTase (Clr4) and an HP1 homolog (Swi6), as well as additional factors. Notably, the HDAC Clr3 is a component of the SHREC complex found at all heterochromatic loci (Sugiyama *et al.* 2007) and is important for heterochromatin formation. Clr3 deacetylase activity has been shown to recruit Clr4 to establish and maintain heterochromatin (Yamada *et al.* 2005).

We found four Neurospora genes with hallmarks of classical histone deacetylases (de Ruijter *et al.* 2003) and named them *hda-1*–*hda-4* (Borkovich *et al.* 2004). As a step in assessing their possible role in DNA methylation, we attempted to disrupt each of the *hda* genes but found indications that one, *hda-3*, is essential for viability. Interestingly, a partial loss-of-function allele, *hda-3*^{RIP1}, resulted in partial loss of DNA methylation. Mutation of two other genes, *hda-2* and *hda-4*, had no discernible effect on DNA methylation. Finally, a null allele of *hda-1* resulted in a regional DNA methylation defect, with some chromosomal regions showing no loss of methylation, some a partial loss, and others an apparent complete loss of methylation. We explored the mechanism of this loss of DNA methylation by investigating histone modification changes in the *hda* mutants.

MATERIALS AND METHODS

Protein sequence alignments: *N. crassa* HDAC protein sequences were aligned against *S. cerevisiae* and *S. pombe* sequences using CLUSTALW, and a PHYLIP rooted phylogenetic tree based on this alignment (Figure 1) was generated at the Biology WorkBench (http://seqtool.sdsc.edu/CGI/BW.cgi). Protein accession numbers are listed in Table 1.

Strains and growth conditions: All *N. crassa* and *S. pombe* strains used in this study are listed in Table 2. Standard conditions were used for *N. crassa* growth and maintenance (Davis 2000). *S. pombe* strains were grown in YES medium (5 g/liter yeast extract, 30 g/liter glucose supplemented with 225 mg/liter adenine, histidine, leucine, uracil, and lysine hydrochloride) to mid-log phase using standard conditions.

Creation of mutants using repeat-induced point mutation: The *hda-1*, *hda-2*, and *hda-3* genes were isolated by PCR amplification of gene fragments using degenerate primers for conserved regions (Table 3) that were used as probes of the Orbach-Sachs cosmid library and Neurospora λ genomic library. The *hda-4* gene was identified by BLAST-searching the Neurospora genome database (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html) using known histone deacetylase domains from *S. cerevisiae*, *Homo sapiens*, and *Drosophila melanogaster* as query sequences.

Genes were subcloned into *his-3* targeting vectors (Margolin *et al.* 1997) and transformed into *his-3* strains N623 (*hda-1* and *-3*), N1673 (*hda-2*), or N1674 (*hda-4*) by electroporation (Margolin *et al.* 1997). His-3$^+$ transformants were selected and crossed to N617 (*hda-1*, *-2*, and *-3*) or N411 (*hda-4*). Genomic DNA from the progeny was analyzed for RFLPs indicative of mutations from repeat-induced point mutation (RIP) (Cambareri *et al.* 1989) by Southern hybridization analyses. Mutant strains were backcrossed to N617 (*hda-1*), N2540 (*hda-3*), or N623 (*hda-2* and *hda-4*). Sequencing of the mutant alleles was performed on strains showing RFLPs.

Southern hybridization analyses: Genomic DNA was isolated following 2 days of growth in Vogel's 2% sucrose media as described (Miao *et al.* 2000). Strains used were *dim-2* (N1877), wild type (N617), *hda-1*^{RIP1} (N2670), Δ*hda-1* (N3152), *hda-2*^{RIP1} (N2627), Δ*hda-2* (N3349), *hda-3*^{RIP1} (N2666), *hda-4*^{RIP1} (N2531), Δ*hda-4* (N3351), Δ*hda-1* (N3610), *qde-2*^{RIP}; *sms-2*^{RIP} (N3406), and Δ*hda-1*; *qde-2*^{RIP}; *sms-2*^{RIP} (N4063 and N4065). All deletion strains were generated by the Neurospora functional genomics program (Colot *et al.* 2006) and provided by the Fungal Genetics Stock Center (FGSC). Approximately 0.5 μg of DNA was digested overnight with the designated restriction endonuclease and fractionated by electrophoresis on 0.8% agarose gels. Transfer to membranes and blotting were performed as previously described (Miao *et al.* 2000). When TSA was used, it was added to a final concentration of 1 μm, and the strains were grown with or without the drug for only 27 hr (Selker 1998). All blots were reprobed to confirm complete DNA digestion (data not shown).

Western blots: Nuclei were isolated as described (Baum and Giles 1986) with the inclusion of HDAC inhibitors TSA (1 μm; Wako) (Selker 1998) and butyrate (50 mm; Sigma) in all buffers. Nuclear proteins were separated by 10% SDS-PAGE. Following transfer to PVDF membrane in 10 mm *N*-cyclohexyl-3-aminopropanesulfonic acid, pH 11, 20% methanol, blots were probed as previously described (Tamaru *et al.* 2003) in PBS including 3% nonfat dry milk powder using the following antibodies: α-H3 (Abcam ab1791), α-H3K4me2 (Upstate 07-030), α-H3 acetyl K9 (Abcam ab4441), α-H3 acetyl K14 (Upstate 06-911), α-H3 acetyl K9/K14 (Upstate 06-599), α-H3 acetyl K18 (Upstate 07-354), α-H3 acetyl K23 (Upstate 07-355), α-H3 acetyl K27 (Upstate), and α-H4 tetra-acetyl (Upstate 06-866). Strains used were wild type (N617), *hda-1* (N2670), *hda-2* (N2627), *hda-3* (N2666), and *hda-4* (N2531).

Chromatin immunoprecipitation: Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (Tamaru *et al.* 2003) with the antibodies listed above and α-H3 trimethyl K9 (Cowell *et al.* 2002) on wild type (N150), *hda-1* (N2849), Δ*hda-1* (N3610), *qde-2*^{RIP}; *sms-2*^{RIP} (N3406), and α*hda-1*; *qde-2*^{RIP}; *sms-2*^{RIP} (N4063 and N4065) strains. HDAC inhibitors described above were included in the ChIP lysis buffer for the ChIP experiments shown in Figure 4. Primer sequences not published elsewhere (Adhvaryu and Selker 2008; Smith *et al.* 2008) are for *am*^{RIP8} (468: GATCCGATGTCACGGACAAG and 469: GCCTGCTCGAACTCGGGCTC).

Two-dimensional gel electrophoresis: Nuclei were isolated as for Western blots above and resuspended in an equal volume of 0.15 m NaCl, 10 mm Tris–Cl (pH 8.0), and 1 mg/ml protamine sulfate. Histones were extracted from the nuclear suspensions as previously described (Green and Do 2009). An

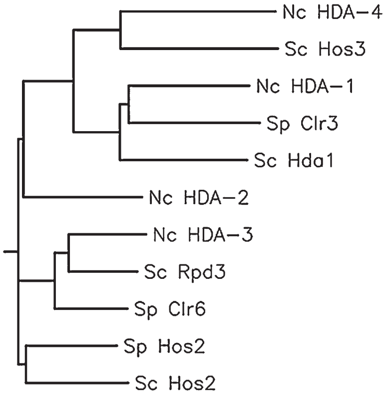

FIGURE 1.—Phylogenetic tree based on a single alignment of predicted protein sequences of *N. crassa* (Nc) and related *S. cerevisiae* (Sc) and *S. pombe* (Sp) HDACs. Even though *N. crassa* HDA-2 is not monophyletic with Hos2 in the tree, pairwise BLAST comparisons indicate that Hos2 is indeed the closest homolog of Neurospora HDA-2.

equal volume of ice-cold 0.4 M H_2SO_4 was added to the nuclear suspension, followed by incubation on ice for 24 hr with occasional mixing. Acid-insoluble material was pelleted by centrifugation at 14,000 × *g* for 10 min. The supernatant was mixed with 100% trichloroacetic acid (TCA) to a final concentration of 20% and incubated on ice for 1 hr. TCA-insoluble proteins were collected by centrifugation at 14,000 × *g* at 4° for 10 min. The protein pellet was resuspended in ice-cold acetone, sonicated in a bath sonicator, and recovered by centrifugation at 14,000× *g* at 4° for 10 min. The acetone precipitate was air-dried at room temperature and then dissolved in acetic acid–urea loading buffer (8.0 M urea, 5% acetic acid, 5% β-mercaptoethanol, 0.2 mg/ml crystal violet) to a concentration of 5 mg/ml. Histones were resolved by two-dimensional gel electrophoresis (2DGE) as described (GREEN and DO 2009). The first-dimension gel contained acetic acid–urea–Triton X-100 (12% polyacrylamide, 5% acetic acid, 6 mM Triton X-100, and 5.0 M urea). The second-dimension gel contained acetic acid–urea (12% acrylamide, 5% acetic acid, and 8 M urea). Gels were stained in 0.05% Coomassie blue R-250, 40% ethanol, and 5% acetic acid; destained in 20% ethanol and 0.5% acetic acid; dried between two cellophane sheets, and scanned with a flatbed scanner.

RESULTS

Characterization of Neurospora *hda* genes: We identified and characterized Neurospora genes encoding HDACs to study the potential role that histone acetylation may play in controlling DNA methylation. At the time that this study began, the *N. crassa* genome sequence was unavailable. We therefore identified Neurospora HDAC genes by PCR amplification using degenerate primers. We isolated three genes, which we named *hda-1*–*hda-3*. When the genome sequence was released, we discovered a fourth HDAC gene, *hda-4*. In addition, seven NAD-dependent deacetylases, named *nst-1*–*nst-7*, are found in the *N. crassa* genome (BORKOVICH *et al.* 2004). The *nst* genes do not appear to play a role in DNA methylation, although at least three of the seven are required for transgene silencing by heterochromatin at Neurospora telomeres (SMITH *et al.* 2008). Since the HDAC inhibitor TSA inhibits some DNA methylation (SELKER 1998), we predicted that increased histone acetylation caused by a deficiency in one or more HDACs would result in reduced DNA methylation. We therefore disrupted each of the four HDAC genes and analyzed the mutants for defects in DNA methylation and histone acetylation.

Creation of Neurospora *hda* mutant strains by RIP: We used RIP to generate mutations in the four *hda* genes by targeting a second copy of each gene to the *his-3* locus and then crossing the duplication strains to induce RIP (SELKER *et al.* 1989). We recovered mutant progeny for each of the four *hda* loci. The *hda-1*^{RIP1} sequence revealed a Q179 to stop mutation (Figure S1). Therefore, the gene product should not include a complete deacetylase domain, implying that this should be a null allele. We found indications that *hda-3* is essential for viability of Neurospora, and successfully isolated an apparent partial loss-of-function allele, *hda-3*^{RIP1}. Most of the ascospores from the cross to induce RIP of *hda-3* were white and nonviable, evidence of lethal mutations. Sequencing of the *hda-3*^{RIP1} allele revealed several nonconservative substitutions in conserved residues within the deacetylase domain and a stop codon downstream of the deacetylase domain at Q385 (Figure S2). The stop codon at Q385 is upstream of a C-terminal domain that is conserved in filamentous fungi and required for *in vitro* deacetylase activity of the *Aspergillus nidulans* homolog, RpdA (TRIBUS *et al.* 2010). We expect that the mutant protein generated

TABLE 1

Histone deacetylases in Neurospora and their closest yeast homologs

N. crassa	*S. cereviseae*	*S. pombe*
HDA-1 (XP_956974)	Hda1 (NP_014377)	Clr3 (NP_595104)
HDA-2 (XP_964451)	Hos2 (NP_011321)	Hos2 (NP_594079)
HDA-3 (XP_964367)	Rpd3 (NP_014069)	Clr6 (NP_595333)
HDA-4 (XP_961839)[a]	Hos3 (NP_015209)	

Protein accession numbers are in parentheses.

[a] The Neurospora database NCU07018 predicts a different start codon, which adds 139 N-terminal amino acids compared to this National Center for Biotechnology Information reference sequence.

TABLE 2

Strains used in this study

Strain	Genotype	Source/reference
N150	*matA*	FGSC 2489
N411	*mat***a** *his-3*	FGSC 6524
N617	*mat***a***; am* ^{RIP8}	Singer *et al.* (1995)
N623	*matA his-3*	FGSC 6103
N1167	*mat***a***; mtr*^{RIP2}*; trp-2*	Rountree and Selker (1997)
N1673	*matA his-3; am*^{132} *inl*	Hays *et al.* (2002)
N1674	*matA his-3; lys-1 am*^{132} *inl; am*^{RIP}*:hph:am*^{RIP}	Hays *et al.* (2002)
N1877	*mat***a** *his-3;* Δ*dim-2::hph*	Kouzminova and Selker (2001)
N2531	*mat***a** *his-3; hda-4*^{RIP1}	This study
N2540	*matA rid*^{RIP1} *his-3*$^{+}$*::ccg-1-hpo*$^{+}$ *sgfp*$^{+}$	This study
N2627	*matA hda-2*^{RIP1}	This study
N2670	*matA; am*^{RIP8} *hda-1*^{RIP1}	This study
N2666	*matA his-3*$^{+}$*::ccg-1-hpo*$^{+}$ *sgfp*$^{+}$*;hda-3*^{RIP1}	This study
N2849	*mat***a***; hda-1*^{RIP1}*; mtr*^{RIP2}*; am*^{RIP8}*; trp-2*	This study
N3152	*mat***a***;* Δ*hda-1::hph*	FGSC 12003
N3349	*matA* Δ*hda-2::hph*	FGSC 11158
N3351	*mat***a***;* Δ*hda-4::hph*	FGSC 11175
N3610	*mat***a***;* Δ*hda-1::hph*	This study
N3406	*matA; qde-2*^{RIP}*; sms-2*^{RIP}	Y. Liu
N3721	*mat***a***; am* ^{RIP8} *mtr*^{RIP2} *trp-2*	This study
N4063	Δ*hda-1::hph; qde-2*^{RIP}*; sms-2*^{RIP}	This study
N4065	Δ*hda-1::hph; qde-2*^{RIP}*; sms-2*^{RIP}	This study
SPG 17	*h*^{90} *leu1-32 ade6-216 his2 ura4*	S. Grewal
SPT 381	*h*$^{+}$ *leu1-32 ade6-216 ura4DS/E, otr1R::ura4*$^{+}$ *clr3*Δ*::kan*	S. Grewal

from the *hda-3*^{RIP1} allele retains some activity because we recovered few progeny and failed to isolate a null allele by RIP, and later, the Neurospora Genome Project (Colot *et al.* 2006) could not isolate a homokaryotic strain containing a knockout allele of this gene (http://www.fgsc.net/). Sequencing of the *hda-2*^{RIP1} and *hda-4*^{RIP1} alleles revealed stop codons upstream of the deacetylase domains in each case (data not shown). We subsequently obtained knockout strains for *hda-1*, *hda-2*, and *hda-4* generated by the Neurospora Genome Project (Table 2).

DNA methylation defects in Neurospora *hda* mutants: To assess the effect of HDAC mutations on DNA methylation, we performed Southern hybridization analyses using restriction enzymes that are sensitive to 5-methyl-cytosine (5mC) in their recognition sites. In Neurospora, DNA methylation is found almost exclusively in RIP-generated, AT-rich regions scattered throughout the genome and concentrated at centromeres and telomeres (Smith *et al.* 2008; Lewis *et al.* 2009). We analyzed the methylation status of several well-studied heterochromatic loci in *hda* mutants by Southern analyses using genomic DNA digested with various restriction enzymes, including the isoschizomers *Sau3AI* (5mC-sensitive) and *DpnII* (5mC-insensitive) (Figure 2, B–E). We included the *dim-2* mutant as a control for complete loss of methylation (Kouzminova and Selker 2001) and compared the results for the various mutants with the effect of TSA treatment (Figure 2, A–C). Analysis of *Bgl*II sites at the 8:A6 region (Figure 2A) revealed that treatment of the control strain with TSA caused a partial loss of methylation, resulting in fragments representing both methylated and unmethylated sites (compare with results for *dim-2* and wild-type strains). Interestingly, DNA from both the *hda-1*^{RIP1} and Δ*hda-1* strains produced a band indicative of loss of methylation. None of the other *hda* mutants showed evidence of altered DNA methylation in the 8:A6 region. TSA treatment caused no detectable loss of methylation in the 8:G3 or 5:B8 regions (Figure 2, B and C), consistent with the previous finding that this HDAC inhibitor causes significant loss of DNA methylation in relatively few loci (see above and Selker 1998). Similarly, we found that loss of methylation in *hda-1* is locus-specific. Striking loss of cytosine methylation was detected at 8:A6 (Figure 2A), 9:E1 (Figure 2E), and RIP'd alleles of *am* and *mtr* (see below), but other regions showed a partial loss of methylation (8:G3 and 5:B8 in Figure 2, B and C). No loss of methylation was found in another set of loci, including 8:F10 (Figure 2D) and 1d21 (see below). In every case, the *hda-1*^{RIP1} mutant and the knockout mutant showed identical methylation phenotypes, consistent with our conclusion that both are null alleles.

A partial loss of methylation in the *hda-3*^{RIP1} mutant was detected at the *am*^{RIP}*:hph:am*^{RIP} and 8:F8 loci but not

TABLE 3

Primer sequences for histone deacetylase detection and amplification

No.	Name	Sequence
1297	hda-1 deg 5′	TCCGGCCTCCCGGNCAYCAYGC
1298	hda-1 deg 3′	CCGTTGCCGTTGTTGAYRTCCCARTC
503	hda-2 deg 5′	CAGTTCAACGTCGGCGANGAYTGYCC
504	hda-2 deg 3′	CCNAARACRATRCAGCAGTTGCTGTAGCAGGAGCC
503	hda-3 deg 5′	CAGTTCAACGTCGGCGANGAYTGYCC
605	hda-3 deg 3′	NCCNGGRAARAAYTCNCCRTA

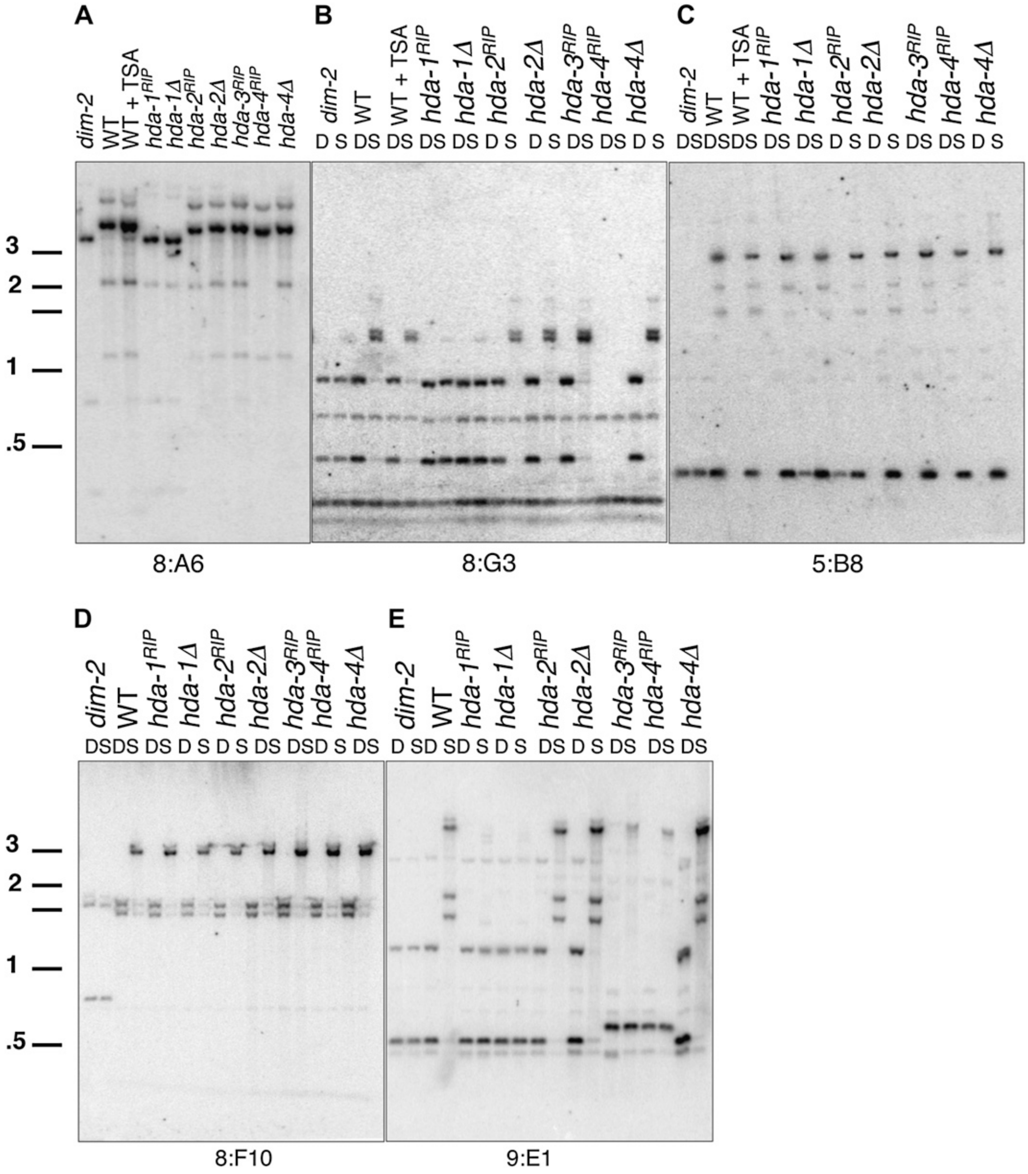

Figure 2.—DNA methylation is lost at some loci in *hda-1*. Southern blots showing genomic DNAs digested with 5mC-sensitive *Bgl*II (A) or with 5mC-insensitive *Dpn*II (lane D) and 5mC-sensitive *Sau3*AI (lane S). Probes are indicated at the bottom of each autoradiogram for panels A–E.

at other regions tested (Figure S3). Methylation at 8:A6, 8:G3, 5:B8, and 8:F10 (Figure 2, A–D) appeared similar to that of wild-type strains. We cannot conclude, however, that *hda-3* is not important for DNA methylation elsewhere because it was not possible to generate a null allele. The $hda\text{-}3^{RIP1}$ and $hda\text{-}4^{RIP1}$ mutants have a different allele of 9:E1 than wild type, accounting for the RFLPs in these two strains (Figure 2E); the $hda\text{-}4^{RIP1}$ mutant lacks the 8:G3 sequence (Figure 2B). The *hda-2* and *hda-4* mutations did not affect DNA methylation at any region tested, and again, the deletion and RIP-mutated alleles gave identical results.

The stabilization of heterochromatin in *S. pombe* involves both the RNAi-mediated RITS complex and the Clr3 HDAC-mediated SHREC complex (Grewal 2010). Previous work from our lab demonstrated that RNAi components in Neurospora are dispensable for the establishment and maintenance of heterochromatin and DNA methylation (Freitag *et al.* 2004b). We considered the possibility that redundant systems, similar to *S. pombe*, were at work maintaining heterochromatin and DNA methylation in Neurospora. To investigate this possibility, we created a strain that was mutant for both of Neurospora's argonaute proteins (Catalanotto *et al.* 2002; D. W. Lee *et al.* 2003; H. C. Lee *et al.* 2010), $qde\text{-}2^{RIP}$ and $sms\text{-}2^{RIP}$, in combination with the *hda-1*Δ allele. We then assayed DNA methylation and histone H3 K9me3 at regions that showed little to no change in the *hda-1*Δ strain alone. DNA methylation at the 5:B8 and 8:F10 regions (Figure S4, A and B) and histone H3 K9me3 at the 8:F10 region (Figure S4C) remained unaltered in the *hda-1*Δ; $qde\text{-}2^{RIP}$;$sms\text{-}2^{RIP}$ triple mutant. These results are consistent with our previous observations that the classical RNAi machinery does not play a role in the maintenance of heterochromatin and DNA methylation in Neurospora.

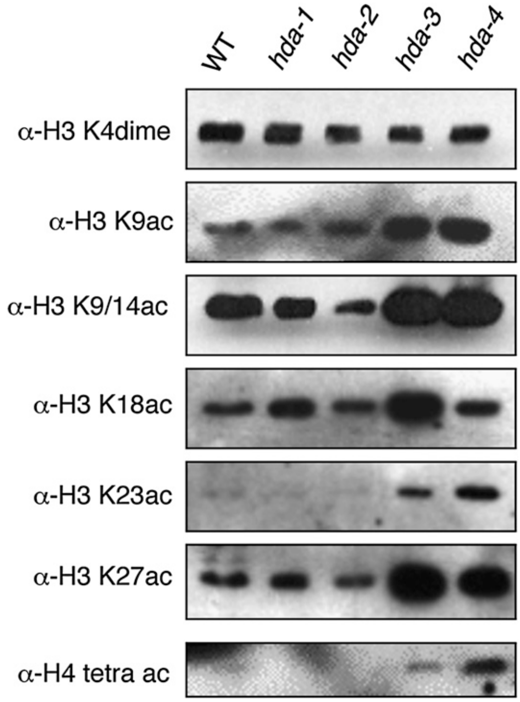

FIGURE 3.—Histone hyper-acetylation in *hda* mutants. Western blots of nuclear proteins from wild type (WT) or from the indicated *hda*^{RIP} mutant strains were probed with the antibody designated at the left of the blots. Antibodies to H3K4me2 (top) and unmodified H3 (not shown) were used to verify equal loading of histones.

Global changes in site-specific acetylation of histones H3 and H4 in Neurospora *hda* mutants revealed by Western blotting: To investigate how *hda* mutations interfere with DNA methylation, we first tested the overall acetylation status at histone residues that can be assessed by Western blotting of nuclear proteins with available antibodies for specific histone H3 and H4 acetylation sites (Figure 3). The *hda-3*^{RIP1} and *hda-4*^{RIP1} mutants showed increased acetylation at every site assayed except at H3 K18 in *hda-4*^{RIP1}, suggesting that Neurospora HDA-3 and HDA-4 have broad specificities on histones H3 and H4. The *hda-2*^{RIP1} mutation, which showed no effect on DNA methylation, caused no significant global increase in acetylation of either H3 or H4 by the Western analysis although there may be a small decrease in acetylation in this mutant at H3 K9/14 and K18. Interestingly, the mutant that showed markedly reduced DNA methylation, *hda-1*^{RIP1}, showed a possible small increase in H3 K18 acetylation but did not show a prominent global change in acetylation with any of the available antibodies against acetylated H3 and H4. Mutation of H3 K18 has no effect on DNA methylation *in vivo* and does not affect DIM-5 activity *in vitro* (K. ADHVARYU, E. BERGE, H. TAMARU, M. FREITAG and E. SELKER, unpublished results).

Local changes in H3 K9/K14 acetylation in Neurospora HDAC mutants revealed by chromatin immunoprecipitation: The Western blots did not provide clear evidence for a change in global acetylation of H3 and H4 that might account for the effect of *hda-1*^{RIP} on DNA methylation at selective chromosomal regions. We therefore turned to ChIP experiments to test for altered acetylation at representative affected loci. ChIP/PCR experiments failed to show increased H3 K18 acetylation at regions that lose DNA methylation (data not shown), but subtle effects were observed elsewhere (Figure 4). We directly compared two regions that are normally methylated, including one that loses methylation (underlined in Figure 4B) and one that is unaffected. PCR amplification of regions with DNA methylation is challenging because these regions are typically repetitive and AT-rich. We therefore generated strains carrying additional methylated low-copy-number regions, RIP'd alleles of *am* (*am*^{RIP8}) and *mtr* (*mtr*^{RIP2}) that we found lost their DNA methylation in an *hda-1*^{RIP1} background (Figure 4A). An additional methylated region unaffected by *hda-1*^{RIP1}, 1d21, was also studied. We performed triplex PCR, including primers to a euchromatic gene as a control (*hH4* or *pcna*) and primers for two methylated regions. Direct comparison of a methylated region to the euchromatic control can give misleading results because global increases in acetylation may also change the level of acetylation at euchromatic regions. Therefore we used triplex PCRs, including a euchromatic region as an additional internal control, but for quantification of enrichment we calculated the ratio of the methylated region that loses methylation (underlined in Figure 4B) to the methylated region that retains methylation (1d21 or 8:F10).

As expected, in wild-type strains, heterochromatic regions showed high H3K9me3 and low H3K4me2, while the euchromatic regions showed the opposite pattern (Figure 4B). Heterochromatic regions that showed loss of DNA methylation in *hda-1*^{RIP} (*am*^{RIP8}, *mtr*^{RIP2}, and 8:G3) showed specific loss of H3K9me3, and regions that retained DNA methylation (1d21 and 8:F10) also retained methylation of H3K9. The *hda-1* mutation had no obvious effect on K4me2. Little change in relative enrichment of H3 K9/K14 acetylation was seen at regions that had lost DNA methylation; the enrichment at *am*^{RIP8} increased from 1.0 to 1.2 relative to 1d21 (Figure 4B, top), and the ratio of H3 K9/K14 acetylation increased from 0.6 to 0.9 at *mtr*^{RIP2} relative to 8:F10 (Figure 4B, middle); the relative enrichment of H3 acetylation at 8:G3 did not increase, but it is noteworthy that while both *am*^{RIP8} and *mtr*^{RIP2} completely lose DNA methylation in *hda-1*^{RIP1} (Figure 4A), 8:G3 shows only a partial loss of DNA methylation (Figure 2B). The slightly increased acetylation at *am*^{RIP8} and *mtr*^{RIP2} was observed only with the antibody that detects H3 acetylated at either K9 or K14. No change was observed with an antibody specific for acetylation of only H3K9 acetylation. This suggests that this modest effect was specific to K14, consistent with results for the closely related *S. pombe* HDAC Clr3 (SUGIYAMA *et al.* 2007), but no specific antibodies were available to confirm this.

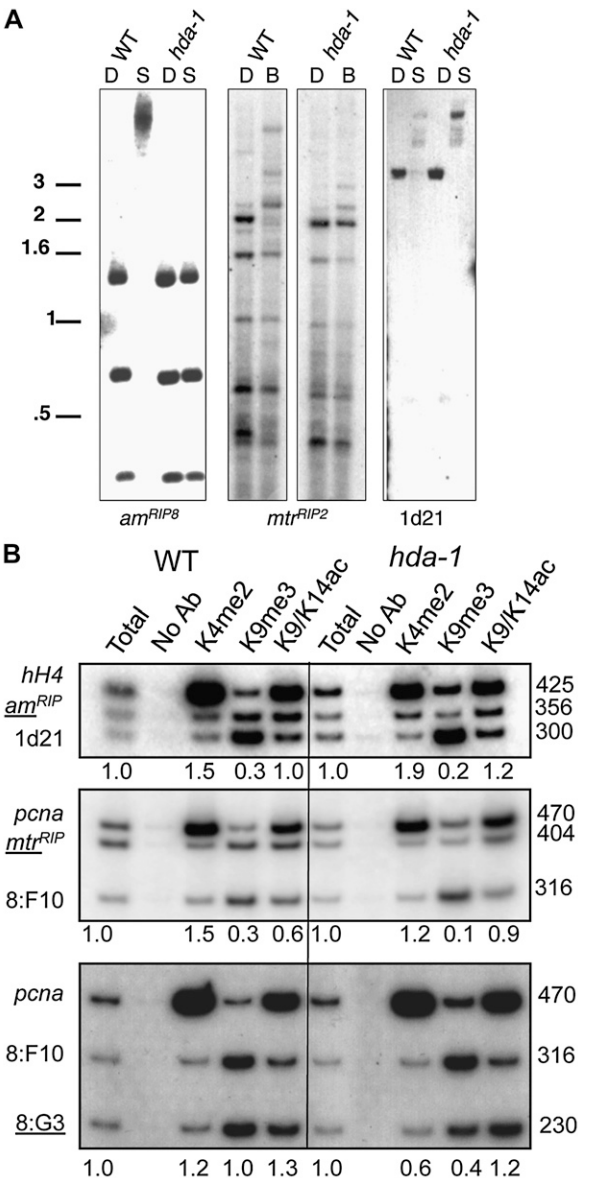

Figure 4.—Chromatin immunoprecipitation shows increased H3 acetylation at regions that lose methylation in *hda-1*^{RIP1}. (A) Southern analysis showing loss of DNA methylation in *hda-1*^{RIP1} at *am*^{RIP8} and *mtr*^{RIP2}, but not 1d21. Strains are N617 (WT) and N2670 (*hda-1*) for *amRIP*^{8} and 1d21 blots and digests are *Dpn*II (D) and *Sau3*AI (S). For the *mtr*^{RIP2} blot, strains are N3721 (WT) and N2849 (*hda-1*), and the 5mC-sensitive isoschizomer *Bfu*CI (B) was used instead of *Sau3*AI. (B) Triplex PCR reactions were performed with ChIP'd DNA as template in wild-type (WT) and *hda-1*^{RIP1} strains, with antibodies specific for H3 K4me2, H3 K9me3, and H3 K9/K14acetyl. *hH4* and *pcna* are euchromatic controls, and the other two regions shown in each panel are methylated in wild type. Regions that lose DNA methylation in *hda-1*^{RIP1} are underlined. Sizes of PCR products are shown on the left, and relative enrichment is shown under the top, middle, and bottom. Relative enrichment is calculated as the ratio of *am*^{RIP8}:1d21, *mtr*^{RIP2}:8:F10, and 8:G3: 8:F10, normalized to this ratio in the total input DNA.

Global changes in histone modification state in Neurospora *hda* mutants revealed by 2DGE: Considering that the effects of the *hda-1* and *hda-3* mutations on sites of acetylation that could be assayed with available antibodies were subtle, we turned to an approach to examine all four core histones of the *hda* mutants for aberrant acetylation. In particular, we employed high-resolution 2DGE to resolve histones according to their relative charge/mass ratios (Green and Do 2009). In this system, charge-altering modifications such as acetylation and phosphorylation reduce migration of the protein spot in both dimensions, yielding derivative species (spots labeled "b," "c," "d," etc. in Figure 5) that migrate diagonally to the top left of the parent spot "a." Recent studies in our laboratories indicate that the origin of most, if not all, derivative spots observed in Neurospora H3 and H2B histones are generated by histone acetylation, as opposed to histone phosphorylation (Anderson *et al.* 2010). Our results revealed a number of effects of the *hda* mutations. First, the results confirmed the small increase in acetylation of H3 detected by Western analysis of the *hda-1*^{RIP1} mutant (Figure 3), as demonstrated by increased intensity of the derivative H3 spot labeled "b" and decreased intensity of the parent H3 spot ("a") in the *hda-1*^{RIP1} mutant (Figure 5C) relative to the wild-type strain (WT; Figure 5A). The shifted spot is indicative of charge neutralization, presumably due to acetylation, resulting in slower mobility in the first and second dimensions.

While little change in the distribution of modified forms was observed for histones H3 and H4 in the *hda-1*^{RIP1} mutant, a dramatic increase in acetylation of histone H2B was indicated by the 2DGE analysis (Figure 5, A and C). Thus, in Neurospora, the primary target of the HDA-1 enzyme appears to be histone H2B rather than histone H3. This observation is consistent with our Western analysis, which failed to detect substantial changes in site-specific modification of histone H3 in the *hda-1* mutant (Figure 3). These results suggest the possibility that the observed decreases in DNA methylation in the *hda-1* mutant may be due to failure of HDA-1 to deacetylate histone H2B. This possibility is consistent with the observation that the *S. cerevisiae* HDA-1 homolog, Hda1p, is active on both H3 and H2B (Wu *et al.* 2001). Although H3K14 is thought to be the pertinent target of the *S. pombe* HDA-1 homolog Clr3 (Bjerling *et al.* 2002; Sugiyama *et al.* 2007), the possibility of effects on histone H2B had not been examined in this organism.

As a step in examining the possible generality of our findings, we analyzed histones from an *S. pombe* Clr3 mutant by 2DGE. As observed in the Neurospora *hda-1* mutant, we found evidence of increased H2B acetylation (Figure 5, B and D). In wild-type *S. pombe*, H2B was predominantly unmodified (Figure 5B) whereas H2B appeared multiply acetylated in the *clr3* mutant (Figure 5D). The major H2B spot, labeled "d," apparently has three charged residues masked, likely by acetylation

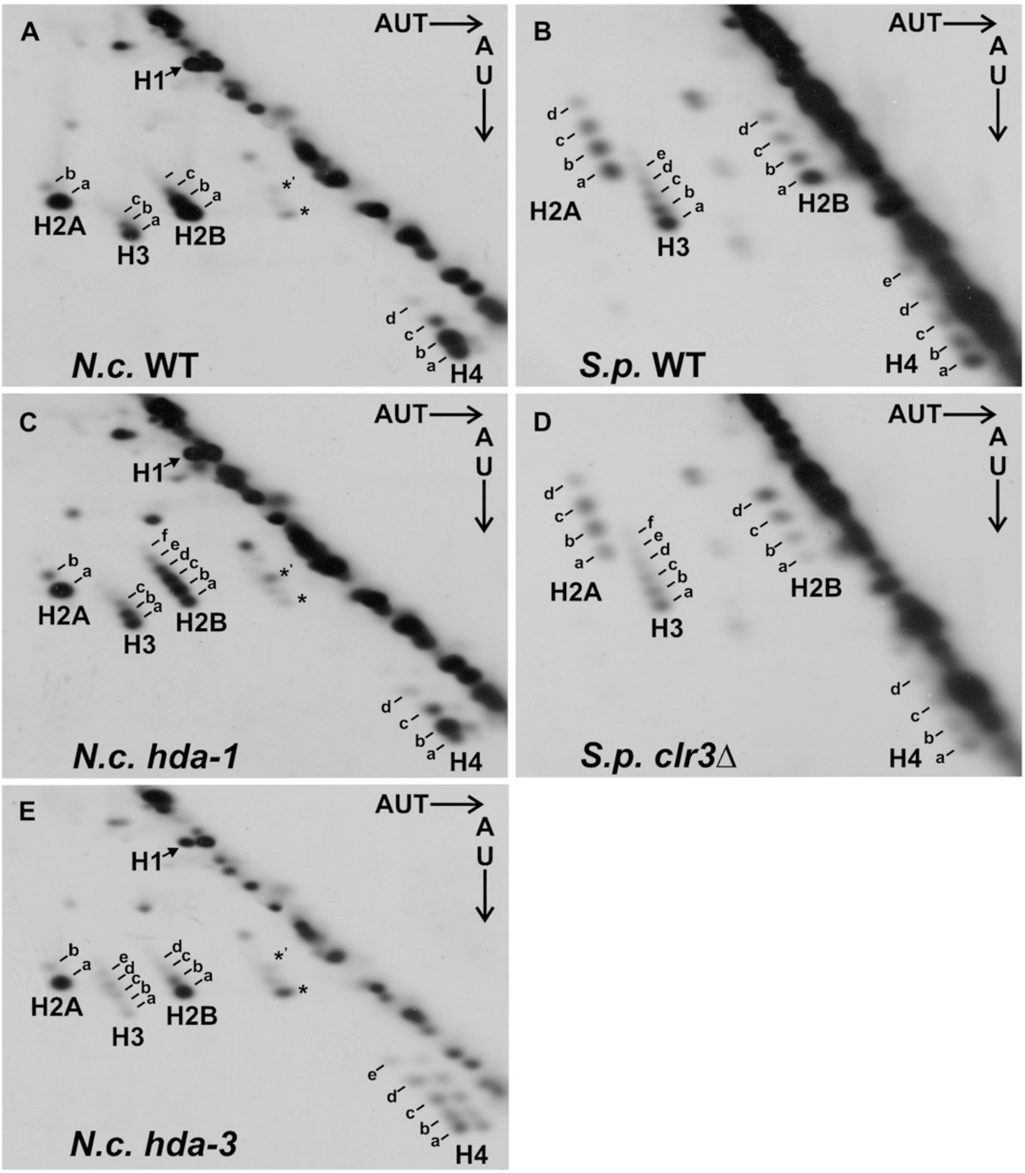

FIGURE 5.—Global changes in histone modification state revealed by two-dimensional gel electrophoresis (2DGE). (A) Wild-type (WT) (N617) Neurospora core histones; all have additional diagonal spots above the bottom parent spot, which represent additional acetylated states of each histone. (B) WT *S. pombe* (SPG 17) histones. (C) Neurospora *hda-1*^{RIP1} mutant (N2670) shows increased acetylation of H2B and a slight increase in H3 acetylation compared to WT (A). (D) *S. pombe clr3* mutant (SPT 381) shows increased H2B acetylation. (E) Neurospora *hda-3*^{RIP1} mutant (N2666) shows more extensive acetylation of H3 and H4 than WT (A). Protein spots are labeled "a," "b," "c," "d," etc. A minor H2B subtype observed in *N. crassa* (labeled *) also appeared to be hyperacetylated in the *hda1* mutant (labeled *').

(ANDERSON *et al.* 2010). In addition, the 2DGE revealed that *S. pombe* Clr3 also targets H2A, as indicated by the redistribution of H2A spots in the *clr3* mutant toward the less mobile derivative spots b, c, and d (Figure 5, B and D).

We also performed 2DGE analysis on the Neurospora *hda-3*^{RIP1} mutant, which also affected DNA methylation, although not as dramatically as *hda-1*^{RIP1} (Figure 2). In contrast to the 2DGE data obtained for Neurospora *hda-1* and *S. pombe* Clr3 (Figure 5C and D, respectively), no global change in acetylation was detected by 2DGE for the H2A or H2B histones in the *hda-3*^{RIP1} mutant (Figure 5E). However, the 2DGE analysis did show a shift in the distribution of acetylation states of histones H3 and H4 in the *hda-3*^{RIP1} mutant, as indicated by the accumulations of di-, tri-, and tetra-acetylated H3 and H4 forms (spots b, c, d, and e, respectively, in Figure 5E). These observations are consistent with our Western analysis of the *hda-3*^{RIP1} strain, which revealed increased acetylation at multiple sites in histone H3 and a modest increase in tetra-acetylation of histone H4. Thus DNA methylation defects in *hda-3*^{RIP1} may be attributed to defective activity of the HDA-3 enzyme.

DISCUSSION

This study followed up an early indication that HDACs play a role in DNA methylation in Neurospora, namely that the HDAC inhibitor TSA caused a selective loss of DNA methylation (SELKER 1998). We identified four genes encoding HDACs in *N. crassa* and disrupted them to assess their possible role in the control of DNA methylation. One of the four genes, *hda-3,* appears to be essential for viability of *N. crassa* and other filamentous fungi (TRIBUS *et al.* 2010). We managed to generate a partial loss-of-function allele, *hda-3*^{RIP1}, by exploiting the genome defense mechanism RIP. Analysis of this mutant suggested that HDA-3 has broad specificity for acetylated lysines on H3 and H4 (Figures 3 and 5) and produced evidence that this HDAC plays a modest role in DNA methylation. The *S. pombe* homolog of HDA-3, Clr6, is also essential for viability (Grewal *et al.* 1998), has broad specificity for acetylated lysines in H3 and H4 (Bjerling *et al.* 2002), and is required to prevent spurious transcription of both genes and heterochromatic repeats (Nicolas *et al.* 2007). Interestingly, *Arabidopsis thaliana* HDA6, which is the closest homolog of Neurospora HDA-3, is required for full RNA-directed DNA methylation (Aufsatz *et al.* 2002) and transcriptional gene silencing (Probst *et al.* 2004). It may be possible to generate temperature-sensitive *hda-3* alleles in Neurospora or to conditionally express *hda-3* to further decrease HDA-3 activity to better elucidate its function in DNA methylation.

We found pronounced DNA methylation defects in mutants of *hda-1*. Indeed, the defects were considerably more extensive than those resulting from TSA treatment. The *S. cerevisiae* and *S. pombe* homologs of HDA-1 have been reported to be specific for H3 K14 (Bjerling *et al.* 2002; Sugiyama *et al.* 2007) and H3 and H2B (Wu *et al.* 2001), respectively. The *S. pombe* homolog of HDA-1, Clr3, is required for recruitment of the H3 K9 HMTase Clr4 to heterochromatin and directly interacts with the HP1 homolog, Swi6 (Yamada *et al.* 2005). Heterochromatin nucleation and spreading requires Clr3 deacetylase activity, Clr4, and Swi6. Moreover, *clr3* mutants have been shown to cause increased acetylation of H3 K14 and increased RNA PolII occupancy at heterochromatic regions (Sugiyama *et al.* 2007). Mutation of *N. crassa hda-1* resulted in a similar, albeit modest, increase in H3 acetylation at the regions of heterochromatin that lost DNA methylation in this mutant (Figure 4B). *N. crassa* HDA-1 may therefore play a similar role in restricting histone acetylation and transcription of heterochromatin, which may then impact DNA methylation.

Our finding of a sizable increase in H2B acetylation in the *hda-1* mutant of Neurospora as well as in the *clr3* mutant of *S. pombe* (Figure 5) raises the interesting possibility that H2B may indirectly impact methylation of H3K9, which is a hallmark of heterochromatin in both organisms and is required for DNA methylation in Neurospora (Tamaru *et al.* 2003). Together with observations on *S. cerevisiae* Hda1 (Wu *et al.* 2001), our findings suggest that H2B is an important and general target of this particular HDAC type. It is noteworthy that there is firm precedent for interplay between modifications on H2B and H3. For example, H2B ubiquitination is required for H3 methylation in yeast (Sun and Allis 2002), and in *A. thaliana*, H2B ubiquitination interferes with H3 K9 methylation and DNA methylation (Sridhar *et al.* 2007). There is no previous evidence for H2B acetylation regulating H3 methylation, but we suggest that this possibility deserves investigation.

We thank Tamir Khalafallah, Jon Murphy, and Jeewong Choi for technical assistance. The *S. pombe* strains were generously provided by Shiv Grewal, and Neurospora strain N3406 was a generous gift from Yi Liu. This work was supported by grants from the National Institutes of Health (GM025690) and the National Science Foundation (MCB-0121383) to E.U.S. and by an American Cancer Society Postdoctoral Fellowship to K.M.S. (PF-04-043-01-GMC).

LITERATURE CITED

Adhvaryu, K. K., and E. U. Selker, 2008 Protein phosphatase PP1 is required for normal DNA methylation in Neurospora. Genes Dev. **22:** 3391–3396.

Anderson, D. C., G. R. Green, K. Smith and E. U. Selker, 2010 Extensive and varied modifications in histone H2B of wild-type and *histone deacetylase 1* mutant *Neurospora crassa*. Biochemistry **49:** 5244–5257.

Aufsatz, W., M. F. Mette, J. Van Der Winden, M. Matzke and A. J. Matzke, 2002 HDA6, a putative histone deacetylase needed to enhance DNA methylation induced by double-stranded RNA. EMBO J. **21:** 6832–6841.

Baum, J. A., and N. H. Giles, 1986 DNase I hypersensitive sites within the inducible *qa* gene cluster of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **83:** 6533–6537.

Bjerling, P., R. A. Silverstein, G. Thon, A. Caudy, S. Grewal *et al.*, 2002 Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. Mol. Cell. Biol. **22:** 2170–2181.

Borkovich, K. A., L. A. Alex, O. Yarden, M. Freitag, G. E. Turner *et al.*, 2004 Lessons from the genome sequence of *Neurospora crassa*: tracing the path from genomic blueprint to multicellular organism. Microbiol. Mol. Biol. Rev. **68:** 1–108.

Cambareri, E. B., B. C. Jensen, E. Schabtach and E. U. Selker, 1989 Repeat-induced G-C to A-T mutations in Neurospora. Science **244:** 1571–1575.

Cameron, E. E., K. E. Bachman, S. Myohanen, J. G. Herman and S. B. Baylin, 1999 Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet. **21:** 103–107.

Catalanotto, C., G. Azzalin, G. Macino and C. Cogoni, 2002 Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in Neurospora. Genes Dev. **16:** 790–795.

Chen, H., J. W. Crabb and J. A. Kinsey, 1998 The Neurospora *aab-1* gene encodes a CCAAT binding protein homologous to yeast HAP5. Genetics **148:** 123–130.

Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew *et al.*, 2006 A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. USA **103:** 10352–10357.

Cowell, I. G., R. Aucott, S. K. Mahadevaiah, P. S. Burgoyne, N. Huskisson *et al.*, 2002 Heterochromatin, HP1 and methylation at lysine 9 of histone H3 in animals. Chromosoma **111:** 22–36.

Cunliffe, V. T., 2008 Eloquent silence: developmental functions of class I histone deacetylases. Curr. Opin. Genet. Dev. **18:** 404–410.

Davis, R. H., 2000 *Neurospora: Contributions of a Model Organism*. Oxford University Press, Oxford.

de Ruijter, A. J., A. H. van Gennip, H. N. Caron, S. Kemp and A. B. van Kuilenburg, 2003 Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem. J. **370:** 737–749.

Freitag, M., P. C. Hickey, T. K. Khlafallah, N. D. Read and E. U. Selker, 2004a HP1 is essential for DNA methylation in Neurospora. Mol. Cell **13:** 427–434.

Freitag, M., D. W. Lee, G. O. Kothe, R. J. Pratt, R. Aramayo *et al.*, 2004b DNA methylation is independent of RNA interference in Neurospora. Science **304:** 1939.

Green, G. R., and D. P. Do, 2009 Purification and analysis of variant and modified histones using 2D PAGE. Methods Mol. Biol. **464:** 285–302.

Grewal, S. I., 2010 RNAi-dependent formation of heterochromatin and its diverse functions. Curr. Opin. Genet. Dev. **20:** 134–141.

Grewal, S. I., M. J. Bonaduce and A. J. Klar, 1998 Histone deacetylase homologs regulate epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. Genetics **150:** 563–576.

Hays, S. M., J. Swanson and E. U. Selker, 2002 Identification and characterization of the genes encoding the core histones and histone variants of *Neurospora crassa.* Genetics **160:** 961–973.

Hollender, C., and Z. Liu, 2008 Histone deacetylase genes in Arabidopsis development. J. Integr. Plant Biol. **50:** 875–885.

Honda, S., and E. U. Selker, 2008 Direct interaction between DNA methyltransferase DIM-2 and HP1 is required for DNA methylation in *Neurospora crassa.* Mol. Cell. Biol. **28:** 6044–6055.

Kouzminova, E., and E. U. Selker, 2001 *dim-2* encodes a DNA methyltransferase responsible for all known cytosine methylation in Neurospora. EMBO J. **20:** 4309–4323.

Kurdistani, S. K., S. Tavazoie and M. Grunstein, 2004 Mapping global histone acetylation patterns to gene expression. Cell **117:** 721–733.

Lee, D. W., R. J. Pratt, M. McLaughlin and R. Aramayo, 2003 An argonaute-like protein is required for meiotic silencing. Genetics **164:** 821–828.

Lee, H. C., L. Li, W. Gu, Z. Xue, S. K. Crosthwaite *et al.*, 2010 Diverse pathways generate microRNA-like RNAs and dicer-independent small interfering RNAs in fungi. Mol. Cell **38:** 803–814.

Lewis, Z. A., S. Honda, T. K. Khlafallah, J. K. Jeffress, M. Freitag *et al.*, 2009 Relics of repeat-induced point mutation direct heterochromatin formation in *Neurospora crassa.* Genome Res. **19:** 427–437.

Margolin, B. S., M. Freitag and E. U. Selker, 1997 Improved plasmids for gene targeting at the *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet. Newsl. **44:** 34–36.

Mateescu, B., P. England, F. Halgand, M. Yaniv and C. Muchardt, 2004 Tethering of HP1 proteins to chromatin is relieved by phosphoacetylation of histone H3. EMBO Rep. **5:** 490–496.

McDonel, P., I. Costello and B. Hendrich, 2009 Keeping things quiet: roles of NuRD and Sin3 co-repressor complexes during mammalian development. Int. J. Biochem. Cell Biol. **41:** 108–116.

Miao, V. P., M. Freitag and E. U. Selker, 2000 Short TpA-rich segments of the zeta-eta region induce DNA methylation in *Neurospora crassa.* J. Mol. Biol. **300:** 249–273.

Nicolas, E., T. Yamada, H. P. Cam, P. C. Fitzgerald, R. Kobayashi *et al.*, 2007 Distinct roles of HDAC complexes in promoter silencing, antisense suppression and DNA damage protection. Nat. Struct. Mol. Biol. **14:** 372–380.

Ou, J. N., J. Torrisani, A. Unterberger, N. Provencal, K. Shikimi *et al.*, 2007 Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. Biochem. Pharmacol. **73:** 1297–1307.

Probst, A. V., M. Fagard, F. Proux, P. Mourrain, S. Boutet *et al.*, 2004 Arabidopsis histone deacetylase HDA6 is required for maintenance of transcriptional gene silencing and determines nuclear organization of rDNA repeats. Plant Cell **16:** 1021–1034.

Robyr, D., Y. Suka, I. Xenarios, S. K. Kurdistani, A. Wang *et al.*, 2002 Microarray deacetylation maps determine genome-wide functions for yeast histone deacetylases. Cell **109:** 437–446.

Rountree, M. R., and E. U. Selker, 1997 DNA methylation inhibits elongation but not initiation of transcription in *Neurospora crassa.* Genes Dev. **11:** 2383–2395.

Selker, E. U., 1998 Trichostatin A causes selective loss of DNA methylation in Neurospora. Proc. Natl. Acad. Sci. USA **95:** 9430–9435.

Selker, E. U., 2004 Genome defense and DNA methylation in Neurospora. Cold Spring Harb. Symp. Quant. Biol. **69:** 119–124.

Selker, E. U., E. B. Cambareri, P. W. Garrett, B. C. Jensen, K. R. Haack *et al.*, 1989 Use of RIP to inactivate genes in *Neurospora crassa.* Fungal Genet. Newsl. **36:** 76–77.

Singer, M. J., B. A. Marcotte and E. U. Selker, 1995 DNA methylation associated with repeat-induced point mutation in *Neurospora crassa.* Mol. Cell. Biol. **15:** 5586–5597.

Sinha, I., M. Wiren and K. Ekwall, 2006 Genome-wide patterns of histone modifications in fission yeast. Chromosome Res. **14:** 95–105.

Smith, K. M., G. O. Kothe, C. B. Matsen, T. K. Khlafallah, K. K. Adhvaryu *et al.*, 2008 The fungus Neurospora crassa displays telomeric silencing mediated by multiple sirtuins and by methylation of histone H3 lysine 9. Epigenetics Chromatin **1:** 5.

Sridhar, V. V., A. Kapoor, K. Zhang, J. Zhu, T. Zhou *et al.*, 2007 Control of DNA methylation and heterochromatic silencing by histone H2B deubiquitination. Nature **447:** 735–738.

Sugiyama, T., H. P. Cam, R. Sugiyama, K. Noma, M. Zofall *et al.*, 2007 SHREC, an effector complex for heterochromatic transcriptional silencing. Cell **128:** 491–504.

Sun, Z. W., and C. D. Allis, 2002 Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. Nature **418:** 104–108.

Tamaru, H., and E. U. Selker, 2001 A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa.* Nature **414:** 277–283.

Tamaru, H., X. Zhang, D. McMillen, P. B. Singh, J. Nakayama *et al.*, 2003 Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa.* Nat. Genet. **34:** 75–79.

Tribus, M., I. Bauer, J. Galehr, G. Rieser, P. Trojer *et al.*, 2010 A novel motif in fungal class 1 histone deacetylases is essential for growth and development of Aspergillus. Mol. Biol. Cell **21:** 345–353.

Wiren, M., R. A. Silverstein, I. Sinha, J. Walfridsson, H. M. Lee *et al.*, 2005 Genomewide analysis of nucleosome density histone acetylation and HDAC function in fission yeast. EMBO J. **24:** 2906–2918.

Wu, J., N. Suka, M. Carlson and M. Grunstein, 2001 TUP1 utilizes histone H3/H2B-specific HDA1 deacetylase to repress gene activity in yeast. Mol. Cell **7:** 117–126.

Yamada, T., W. Fischle, T. Sugiyama, C. D. Allis and S. I. Grewal, 2005 The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. Mol. Cell **20:** 173–185.

Zilberman, D., 2008 The evolving functions of DNA methylation. Curr. Opin. Plant Biol. **11:** 554–559.

Communicating editor: A. P. Mitchell

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.123315/DC1

H2B- and H3-Specific Histone Deacetylases Are Required for DNA Methylation in *Neurospora crassa*

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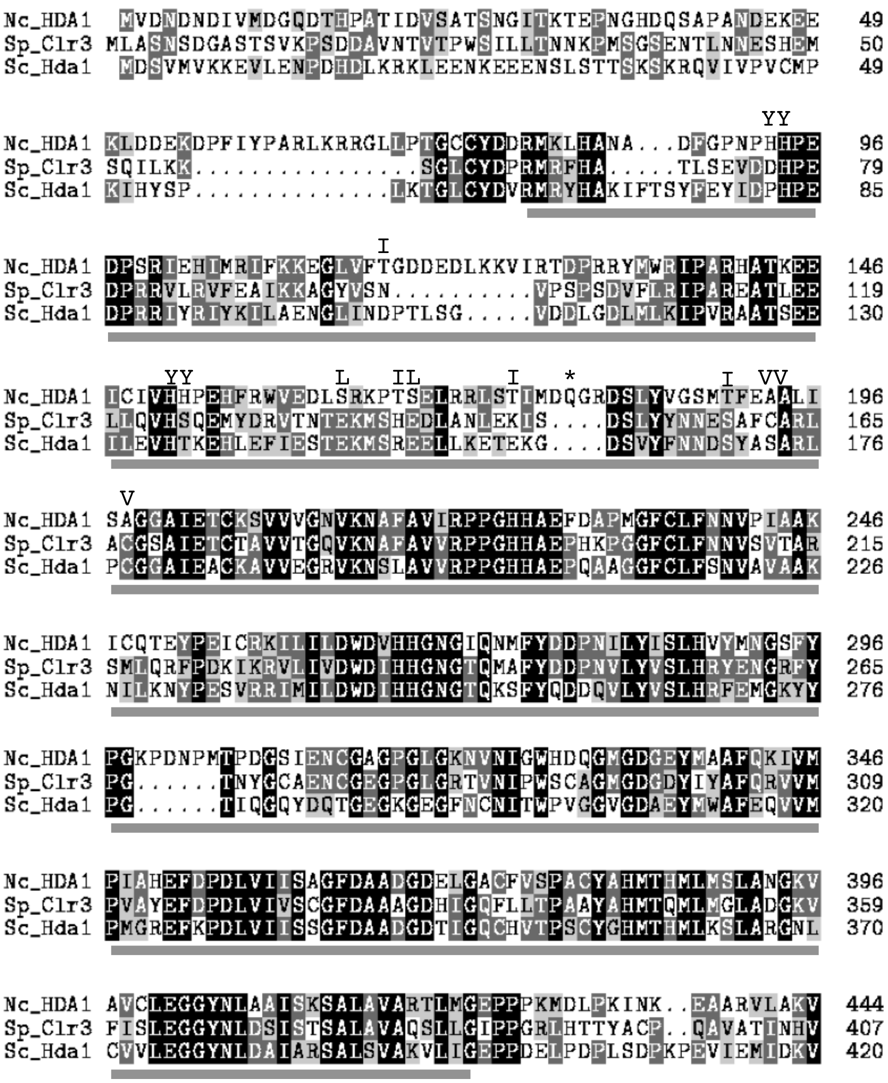
DOI: 10.1534/genetics.110.123315



```
                                                      YY
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Sp_Clr3 MLASNSDGASTSVKPSDDAVNTVTPWSILLTNNKPMSGSENTLNNESHEM   50
Sc_Hda1  MDSVMVKKEVLENPDHDLKRKLEENKEEENSLSTTSKSKRQVIVPVCMP   49

                                                      YY
Nc_HDA1 KLDDEKDPFIYPARLKRRGLLPTGCCYDDRMKLHANA...DFGPNPHHPE   96
Sp_Clr3 SQILKK................SGLCYDPRMRFHA.....TLSEVDDHPE   79
Sc_Hda1 KIHYSP..............LKTGLCYDVRMRYHAKIFTSYFEYIDPHPE   85

                           I
Nc_HDA1 DPSRIEHIMRIFKKEGLVFTGDDEDLKKVIRTDPRRYMWRIPARHATKEE  146
Sp_Clr3 DPRRVLRVFEAIKKAGYVSN..........VPSPSDVFLRIPAREATLEE  119
Sc_Hda1 DPRRIYRIYKILAENGLINDPTLSG.....VDDLGDLMLKIPVRAATSEE  130

            YY          L   IL      I   *          I  VV
Nc_HDA1 ICIVHHPEHFRWVEDLSRKPTSELRRLSTIMDQGRDSLYVGSMTFEAALI  196
Sp_Clr3 LLQVHSQEMYDRVTNTEKMSHEDLANLEKIS....DSLYYNNESAFCARL  165
Sc_Hda1 ILEVHTKEHLEFIESTEKMSREELLKETEKG....DSVYFNNDSYASARL  176

         V
Nc_HDA1 SAGGAIETCKSVVVGNVKNAFAVIRPPGHHAEFDAPMGFCLFNNVPIAAK  246
Sp_Clr3 ACGSAIETCTAVVTGQVKNAFAVVRPPGHHAEPHKPGGFCLFNNVSVTAR  215
Sc_Hda1 PCGGAIEACKAVVEGRVKNSLAVVRPPGHHAEPQAAGGFCLFSNVAVAAK  226

Nc_HDA1 ICQTEYPEICRKILILDWDVHHGNGIQNMFYDDPNILYISLHVYMNGSFY  296
Sp_Clr3 SMLQRFPDKIKRVLIVDWDIHHGNGTQMAFYDDPNVLYVSLHRYENGRFY  265
Sc_Hda1 NILKNYPESVRRIMILDWDIHHGNGTQKSFYQDDQVLYVSLHRFEMGKYY  276

Nc_HDA1 PGKPDNPMTPDGSIENCGAGPGLGKNVNIGWHDQGMGDGEYMAAFQKIVM  346
Sp_Clr3 PG......TNYGCAENCGEGPGLGRTVNIPWSCAGMGDGDYIYAFQRVVM  309
Sc_Hda1 PG......TIQGQYDQTGEGKGEGFNCNITWPVGGVGDAEYMWAFEQVVM  320

Nc_HDA1 PIAHEFDPDLVIISAGFDAADGDELGACFVSPACYAHMTHMLMSLANGKV  396
Sp_Clr3 PVAYEFDPDLVIVSCGFDAAAGDHIGQFLLTPAAYAHMTQMLMGLADGKV  359
Sc_Hda1 PMGREFKPDLVIISSGFDAADGDTIGQCHVTPSCYGHMTHMLKSLARGNL  370

Nc_HDA1 AVCLEGGYNLAAISKSALAVARTLMGEPPPKMDLPKINK..EAARVLAKV  444
Sp_Clr3 FISLEGGYNLDSISTSALAVAQSLLGIPPGRLHTTYACP..QAVATINHV  407
Sc_Hda1 CVVLEGGYNLDAIARSALSVAKVLIGEPPDELPDPLSDPKPEVIEMIDKV  420
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FIGURE S1.— *hda-1* RIP mutations. Alignment of the N-terminal end of *N. crassa* HDA-1, *S. pombe* Clr3, and *S. cerevisiae* Hda1 with amino acid changes encoded by the hda-1^{RIP1} gene shown above. The HDAC domain is indicated by the grey bar.

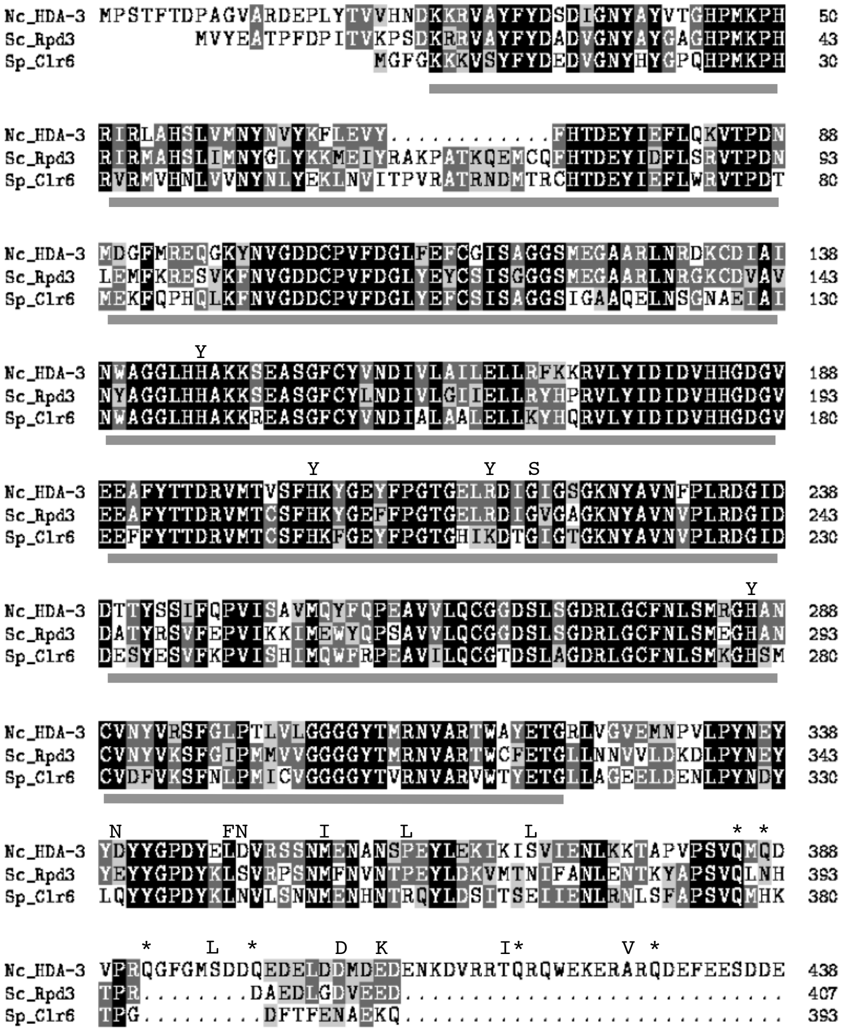

FIGURE S2.— *hda-3* RIP mutations. Alignment of the N-terminal end of *N. crassa* HDA-3, *S. pombe* Clr6, and *S. cerevisiae* Rpd3 with amino acid changes encoded by the *hda-3*^{RIP1} gene shown above. The HDAC domain is indicated by the grey bar.

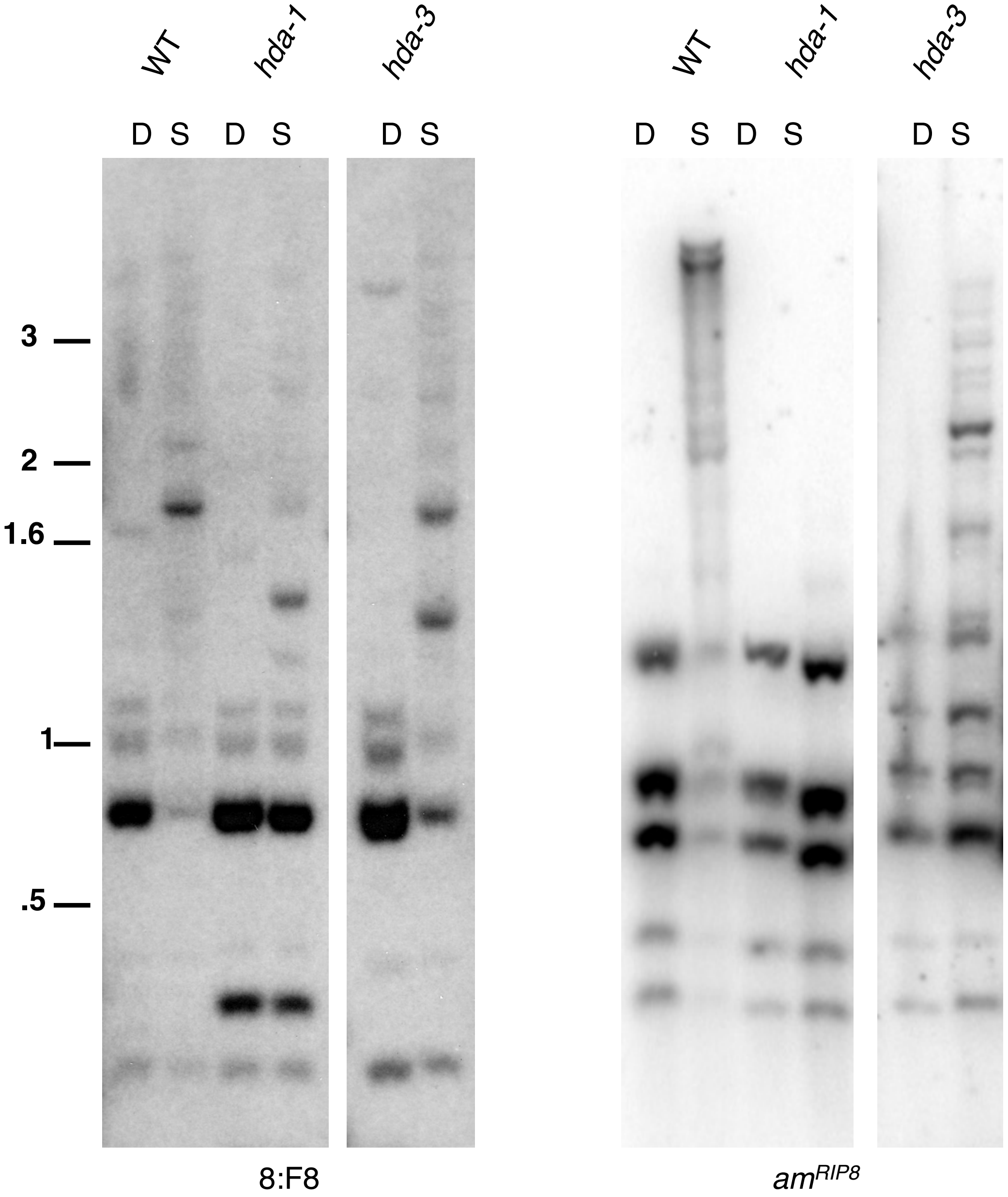

FIGURE S3.— Loss of DNA methylation in *hda-1*^{RIP1} and *hda-3*^{RIP1}. Southern blots showing genomic DNAs digested with 5mC-insensitive *DpnII* (D) and 5mC-sensitive *Sau3AI* (S). Probes are indicated at the bottom of each blot. Strains are N617 (WT), N2670 (hda-1), and N2666 (hda-3).

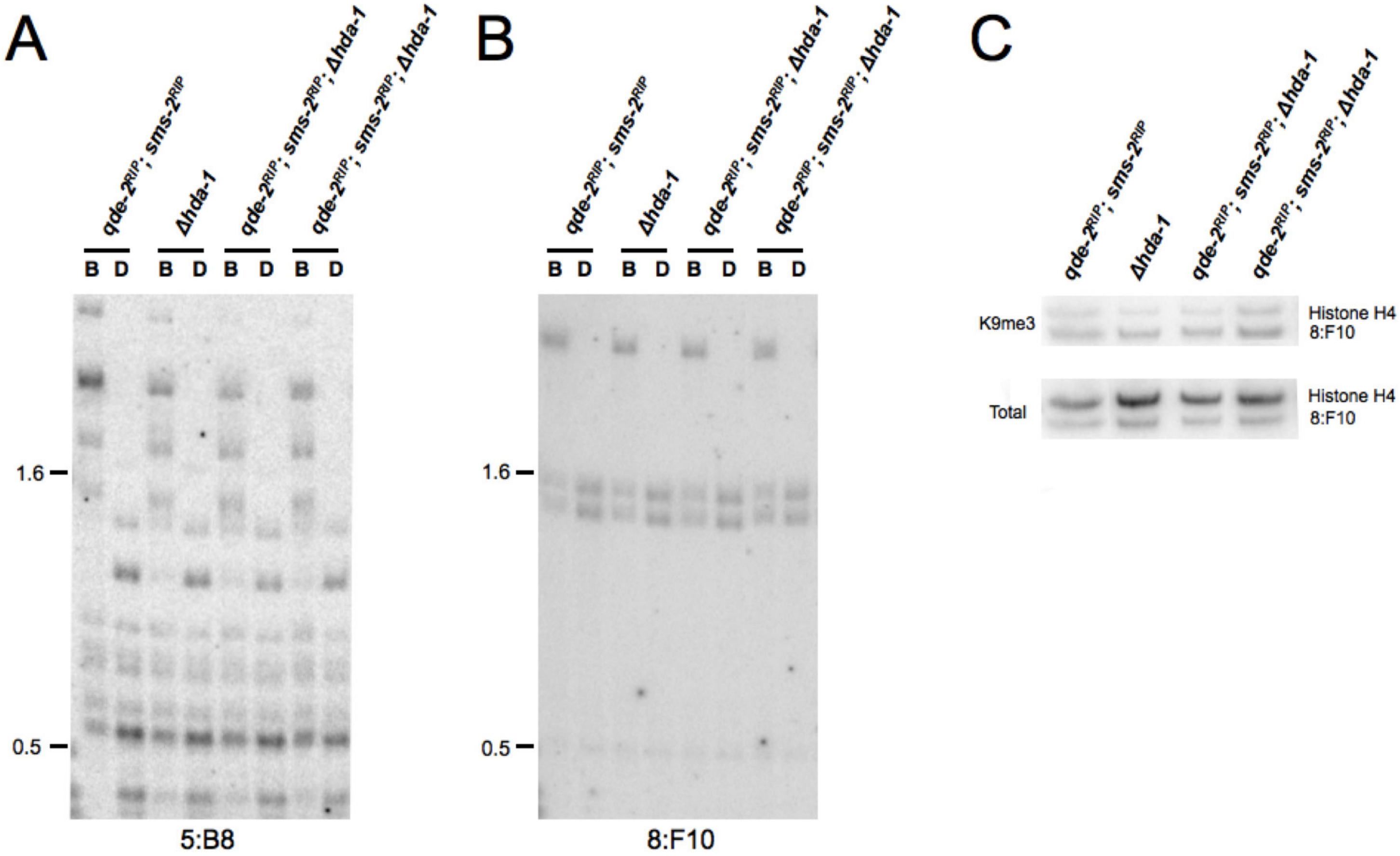

FIGURE S4.— DNA methylation and H3K9me3 remain unchanged in hda-1; qde-2$^{\text{RIP}}$; sms-2$^{\text{RIP}}$ triple mutant at regions that retain DNA methylation in hda-1 mutant. (A) and (B). Southern analysis showing DNA methylation of the 5:B8 and 8:F10 regions in parental strains, N3406 (qde-2$^{\text{RIP}}$; sms-2$^{\text{RIP}}$) and N3610 (Δhda-1::hph), and two representative hda-1; qde-2$^{\text{RIP}}$; sms-2$^{\text{RIP}}$ triple mutant progeny (N4063 and N4065). DNAs were digested with either the 5mC-sensitive enzyme *BfuCI* (B) or its 5mC-insensitive isoschizomer *DpnII* (D). (C). ChIP analysis using an antibody specific for histone H3 K9me3 showing similar levels of enrichment at the 8:F10 region. Either H3 K9me3 ChIPd DNA or total (1:10) DNA was used as template in duplex PCR reactions performed with primer sets for the histone H4 gene (euchromatic control) and the 8:F10 region.