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Dual chromatin recognition by the histone deacetylase complex HCHC is required for proper DNA methylation in *Neurospora crassa*

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DNA methylation, heterochromatin protein 1 (HP1), histone H3 lysine 9 (H3K9) methylation, histone deacetylation, and highly repeated sequences are prototypical heterochromatic features, but their interrelationships are not fully understood. Prior work showed that H3K9 methylation directly links DNA methylation and histone deacetylation, and histone acetyltransferase (HAT) complexes are recruited directly by H3K9me3 (12), which is recognized and bound by the chromodomain (CD) of heterochromatin protein 1 (HP1) (13). HP1 is the primary mediator of de novo DNA methylation de novo (15, 18, 19). We identified DIM-5, DIM-7, and DIM-9, which are members of the KMT complex, DCDC (DIM-5/7/9/CUL4-DDB1 complex), which is required for H3K9me3 and appears to operate by a two-step mechanism: DIM-7-dependent DIM-5 recruitment and CUL4/DDB1/DIM-9-dependent catalysis by the KMT DIM-5 (20, 21).

We previously identified a histone deacetylase (HDAC) complex, HCHC, which contains HP1, CD protein 2 (CDP-2), the DNA methyltransferase (DNMT) DIM-2 is recruited by HP1 through the chromoshadow domain of HP1 and two PxVxL-like motifs in DIM-2 (14).

In *N. crassa*, H3K9me3, HP1, and DNA methylation are colocalized and together define the regions of constitutive heterochromatin (15). The centromere regions, generally rich in transposon relics, account for the largest regions of constitutive heterochromatin, but telomeres and interstitial islands of transposon relics also have some features of heterochromatin. Most of these regions are AT-rich as a result of the genome defense system RIP (repeat-induced point mutation). RIP detects duplicated sequences and induces G:C to A:T mutations in these regions during the sexual phase of the *N. crassa* life cycle (15–17). The resulting AT-rich sequences serve as potent signals for triggering H3K9me3 and DNA methylation de novo (15, 18, 19). We identified DIM-5, DIM-7, DIM-9, CUL4 (cullin 4), and DDB1 (DNA damage-binding protein 1) as components of a KMT complex (20, 21). Dual chromatin recognition by the histone deacetylase complex HCHC is required for proper DNA methylation.

DNA methylation | H3K9 methylation | histone deacetylation | heterochromatin | HP1

DNA methylation is an epigenetic mechanism involved in fundamental biological processes such as transcriptional regulation, genome defense, X chromosome inactivation, and genomic imprinting (1–4). In mammals, patterns of DNA methylation are established during embryonic development and are maintained during subsequent cell divisions (5). Abnormal DNA methylation is associated with human disease, including cancer (6, 7), but the events leading to abnormal DNA methylation are not well understood. DNA methylation is a fundamental biological process that plays vital roles in genome function. Both hypo- and hypermethylation of DNA are associated with human diseases, including cancers, but the underlying mechanisms are not well understood. Using the filamentous fungus *Neurospora crassa*, one of the simplest eukaryotes with DNA methylation, we report a DNA methylation pathway that depends partially on the histone deacetylase complex HCHC (heterochromatin protein 1 (HP1)–chromodomain protein 2 (CDP-2)–histone deacetylase 1 (HDA-1)–associated protein (CHAP)). Genome-wide DNA methylation analysis revealed both hypo- and hyper-DNA methylation in strains with defective HCHC components. We show the interrelationship of HCHC components and genetically dissect the proteins to define domains critical for proper DNA methylation and centromeric silencing. This work provides insights into the crosstalk between DNA methylation and histone modifications.

### Significance

**Modifications of chromatin proteins (e.g., histones) and DNA play vital roles in genome function. Both hypo- and hypermethylation of DNA are associated with human diseases, including cancers, but the underlying mechanisms are not well understood. Using the filamentous fungus Neurospora crassa, one of the simplest eukaryotes with DNA methylation, we report a DNA methylation pathway that depends partially on the histone deacetylase complex HCHC (heterochromatin protein 1 (HP1)–chromodomain protein 2 (CDP-2)–histone deacetylase 1 (HDA-1)–associated protein (CHAP)).** Genome-wide DNA methylation analyses revealed both hypo- and hyper-DNA methylation in strains with defective HCHC components. We show the interrelationship of HCHC components and genetically dissect the proteins to define domains critical for proper DNA methylation and centromeric silencing. This work provides insights into the crosstalk between DNA methylation and histone modifications.


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HDAC HDA-1, and a CDP-2/HDA-1–associated protein, CHAP (22). The HCHC complex works in parallel with the DNMT complex DIM-2/HP1 to establish and maintain normal heterochromatin. In addition, the HCHC complex indirectly maintains proper DNA methylation at regions with moderate and heavy modification by RIP, which respectively show hypo- and hypermethylation in cdp-2, hda-1, or chap mutants (22). Here we described detailed functional interrelationships and domain functions of the components of the HCHC complex.

Results

HCHC Plays an Important Role in Centromere Function. We previously demonstrated that mutants lacking HP1, but not DIM-2, exhibit sensitivity to the microtubule inhibitor thiabendazole (TBZ) and suffer from chromosome segregation defects (20). Because HP1 is present in both the HCHC and DIM-2/HP1 complexes (22) and because centromere regions are hypermethylated in mutants defective in components of HCHC (22), we wished to test if mutants lacking other components of HCHC show these hpo (HP1 gene) phenotypes. We found that the hda-1, cdp-2, and chap mutants did not display sensitivity to CPT comparable to that observed for the hpo mutant (Fig. 4A), suggesting that HP1 has functions other than its role in the HCHC and HP1–DIM-2 complexes. Like hpo strains, mutants lacking HDA-1 exhibited strong sensitivity to TBZ, whereas cdp-2 and chap mutants showed an intermediate level of TBZ sensitivity (Fig. 4A). Like hpo strains but unlike dim-2 strains, all the HCHC mutants showed numerous chromosome bridges (Fig. 1B) (20). These findings fit with our prior observation that HCHC mutants show centromere silencing defects (22) and strengthen the conclusion that HCHC is important for centromere function.

Whole-Genome Bisulfite Sequencing Analysis of the HCHC Mutants. DNA methylation of HCHC mutants was previously assessed in selected genomic regions by Southern hybridization and methylated DNA immunoprecipitation analyses (15). To extend our understanding of the role of HCHC, we carried out whole-genome bisulfite sequencing (WGBS) of cdp-2, chap, and hda-1 mutants. Consistent with our prior analyses (22), a heat map display of the WGBS data revealed both hypomethylated and hypermethylated regions in the three HCHC mutants (Fig. S1). In addition, when methylated regions were sorted by increasing size, we found that shorter regions, which are generally heavily methylated in wild-type HCHC (average of 47.6% of C residues are methylated in the 50 shortest regions), tend to show significantly reduced methylation in the HCHC mutants (averages of 13.9, 14.8, and 22.7% in cdp-2, hda-1, and chap mutants, respectively) (Fig. 2A). Conversely, longer regions, most notably centromeres, are more lightly methylated in wild-type strains (average of 25.9% in the longest 50 regions) but tend to show moderately more methylation in the HCHC mutants (average of 30.7, 32.0, and 35.0% in hda-1, cdp-2, and chap mutants, respectively) (Fig. 2A and B). Sequences near telomeres that are normally methylated were found to lose methylation in the mutants (Fig. 2B and Fig. S1). In addition, sequences with a low combined RIP index (CRI) (17) tend to show reduced methylation in the mutants, whereas sequences with higher CRI scores show increased methylation (Fig. 2C). The borders of normally methylated regions typically lose methylation and show a contraction of boundary methylation in the HCHC mutants (Fig. 2D).

The CD of HP1 but Not That of CDP-2 Is Required for HCHC Complex Function. We previously demonstrated that the CD of CDP-2 efficiently binds to H3K9me3 in vitro (22). Considering that the HCHC complex harbors two proteins containing this domain, we wished to investigate the possibility that the chromodomains might be partially or fully redundant. To do so, we generated constructs to produce epitope-tagged proteins bearing mutations changing or deleting residues known to be critical for the CD function (CDP-2ΔCD, HP1ΔCD), but not targeted to the C-terminal 3×FLAG epitope, and expression was driven by the native cdp-2 promoter. We inserted the constructs at the his-3 locus in a cdp-2 mutant strain and confirmed that their expression levels were all comparable to that in the wild-type strain (Fig. S2A). Curiously, all the cdp-2 constructs bearing CD mutations rescued the cdp-2 defects, namely region-specific hypomethylation (Fig. 3B), global hypermethylation (e.g., at centromere regions) (Fig. S2B), and derepression of centromere silencing (Fig. S2C), suggesting that the CD of CDP-2 is dispensable.

These findings did not eliminate the possibility that the CDP-2 CD has a redundant function, perhaps with HP1. We therefore generated C-terminal HAT-FLAG epitope-tagged HP1 constructs, similar to those created for CDP-2, that contain mutations changing or deleting residues critical for HP1 CD function (hpoΔCD and hpoΔACD) (Fig. S3A). These hpo promoter-driven constructs were inserted at the his-3 locus in a hpo mutant strain. DNA methylation was entirely lost in the Δhpo transformation host strain but was fully restored with the his-3 targeted hpoWT construct (Fig. S3B). Surprisingly, both the HP1 CD mutant constructs, hpoΔACD and hpoΔCD, restored a low level of DNA methylation, indicating that the CD region is not absolutely required for DNA methylation. Similarly, these CD mutants partially alleviated sensitivity to TBZ (Fig. S3C). However, the mutants still showed sensitivity to CPT and defective centromeric silencing (Fig. S3 C and D).

The CDP-2 and HP1 CD mutations described above were expressed at the his-3 locus. To test the effect of simultaneously compromising the chromodomains of both CDP-2 and HP1, we inserted the CD-deletion constructs at their respective endogenous loci (Fig. 3). The new hpo constructs were tagged with the LexA DNA-binding domain (LexADBD) epitope at the C terminus. We confirmed that the strain carrying the tagged hpoWT construct exhibited normal DNA methylation, indicating that the tagged protein was functional (Fig. 3D). As in our results described above (Fig. S3B), we found that deletion of the HP1 CD, unlike the loss of the whole protein (13), resulted in reduced DNA methylation.

Fig. 1. HCHC is important for centromere function. (A) Serial dilutions of conidia from each of the indicated strains were spot-tested on medium with or without TBZ or CPT. Strains: N3753, N4922, 775, 903, and 949. (B) The distribution of the nuclear marker H2A-GFP in growing hyphae in wild-type, cdp-2, hda-1, and chap strains were examined microscopically. The frequency of observed chromatin bridges and the total number of nuclei are shown beside each representative micrograph. Strains: N5015, N5017, N5024, and N5026.
rather than a complete loss of DNA methylation (Fig. 3D). Deletion of the CDP-2 CD did not accentuate the DNA methylation defect of the HP1 CD mutant (Fig. 3E), suggesting that, despite its ability to bind methylated H3K9 in vitro (22), the CDP-2 CD does not act redundantly with the HP1 CD.

**CDP-2 Interacts Directly with the Chromoshadow Domain of HP1.** To gain insights into how HCHC operates without the CDP-2 CD, we investigated the organization of the HCHC subunits and tested the function of their prominent domains (22). We performed a yeast two-hybrid assay and found that HP1 and CDP-2 interact directly and that this interaction requires the chromoshadow domain of HP1 (Fig. 4A). CDP-2 did not interact with the HP1 Y244E mutant, which has a single amino acid substitution in the HP1 chromoshadow domain that prevents both dimerization of HP1 and interaction with the DIM-2 tandem PxVxL-like motifs (14, 23). To determine which CDP-2 sequences interact with the HP1 chromoshadow domain, we generated and tested a series of CDP-2 fragments. We found that amino acids 8–24 of CDP-2 are sufficient for binding HP1 (Fig. S4A). Inspection of the CDP-2 sequence revealed a PxVxL-like motif, (I/F/V)x(I/V)x(I/L/V), at amino acids 14–18 that is conserved among filamentous fungi (Fig. S4B). We generated and tested a mutant construct with alanines substituted for conserved residues at 14 and 15 (IE/AA) in the motif and found that the change abolished interaction with HP1 (Fig. 4B and Fig. S4A). To verify the specific interaction, we deleted a second PxVxL-like motif, ΔPPITL, found at amino acids 33–37, adjacent to the first PxVxL-like motif, and confirmed that it did not abolish the interaction (Fig. S4A). We next created the corresponding CDP-2 mutant strains and tested this interaction in vivo. Each protein included a 3×FLAG epitope tag, and expression was driven by the endogenous promoter. In line with our yeast two-hybrid results, coimmunoprecipitation (co-IP) experiments revealed that the IE/AA mutation abolished interaction with HP1 (Fig. 4C). Interestingly, the ΔPPITL mutation also eliminated interaction with HP1 in vivo even though it did not in vitro (Fig. 4C and Fig. S4A), suggesting CDP-2 might be similar to DIM-2 in requiring tandem PxVxL-like motifs to interact with HP1 (14). Together, these results support a model in which the N-terminal fragment of CDP-2 interacts directly with the chromoshadow domain of HP1.

Fig. 2. WGBS analysis of HCHC mutants. (A) Heat map analyses showing the relative level of 5mC for all methylated regions, sorted from shortest to longest, for wild-type strains and the HCHC mutants. (B) Heat map analyses showing the relative level of 5mC at centromeres and methylated regions within 100 kb of telomeres for wild-type strains and HCHC mutants. (C) The CRI (x axis) and average methylation level (y axis) were calculated for 500-bp windows across the genome (Materials and Methods) and then were plotted for wild-type strains and each of the HCHC mutants. A mutant lacking HP1 (Δhpo) is used as a control for complete loss of DNA methylation. (D) Average methylation levels were calculated for 50-bp windows across the borders of methylated regions (Materials and Methods) and then were plotted for wild-type strains and HP1 mutants. Strains: N3752, N3615, N3612, N3435, and N4922.
CDP-2 and CHAP Interact Directly with HDA-1. We further used the yeast two-hybrid assay to ask how other components of HCHC interact. We found that, in addition to interacting with HP1, CDP-2 interacts with HDA-1 but not with CHAP (Fig. 4A). Similarly, HDA-1 interacts with CHAP but not with HP1, and CHAP does not interact directly with HP1 (Fig. 4A). To identify the protein regions involved in the interactions, we generated and tested a series of CDP-2, HDA-1, and CHAP fragments. The experiments revealed that amino acids 24–54 of CDP-2 are sufficient for binding HDA-1 and that deletion of the PxVxL-related motif PPITL (amino acids 33–37) abolished its interaction with HDA-1 (Fig. 4B and Fig. S4C). In vivo co-IP analysis of this interaction, using the IE/AA and ΔPPITL constructs described above, revealed that only the PPITL motif is required for CDP-2’s interaction with HDA-1 and that the IE/AA mutation had no effect on their interaction (Fig. 4D). Further yeast two-hybrid analysis showed amino acids 87–474 of the HDA-1 HDAC domain were sufficient for binding CDP-2 (Fig. 4B and Fig. S4D), and amino acids 478–744 of the HDA-1 Arb2 (arginone-binding protein 2) domain are sufficient for its direct interaction with CHAP (Fig. 4B and Fig. S4E). To test whether the AT-hook and zinc finger motifs of CHAP are involved in the interaction between HDA-1 and CHAP, we made CHAP point mutants [first AT-hook (ATH1); second AT-hook (ATH2); first zinc finger (Zf1); and second zinc finger (Zf2)] (Fig. S5A) and found that the Zf1 of CHAP is important for its interaction with HDA-1 (Fig. 4B and Fig. S4F). Unfortunately, we were unable to validate the roles of CHAP’s zinc fingers in vivo, because mutant constructs produced unstable CHAP protein (Fig. S5B). The yeast two-hybrid interactions of all the components of HCHC are summarized in Fig. 4B.

CDP-2 Recruits HDA-1 HDAC Activity to H3K9me3 Regions. Based on the interaction map among the HCHC components (Fig. 4B), we hypothesized that CDP-2 might simply serve as a tether between HP1 and HDA-1, facilitating deacetylation of histones marked with H3K9me3. To test this idea, we first carried out co-IP experiments on extracts from strains with epitope-tagged HDA-1 and HP1 in cdp-2-null mutant strains and found that interactions between HDA-1 and HP1 indeed depend on CDP-2 (Fig. S6A). Interestingly, the interaction between HDA-1 and HP1 occurred in a dim-5 mutant, indicating that formation of the HCHC complex can occur before HP1 binding to H3K9me3 (Fig. S6A). To elucidate further the role of CDP-2 in tethering, we performed DamID (DNA adenine methyltransferase identification) by generating wild-type and cdp-2 mutant strains expressing HDA-1–Dam. DamID uses DpnI (which cuts specifically at adenine-methylated GATC sites) and DpnII (which cuts at GATC sites without adenine methylation) to assess adenine methylation catalyzed by Dam. Fusion proteins and can provide information on the genomic localization of proteins that are not detectable by ChIP (21, 24). At all heterochromatic regions tested, expression of HDA-1–Dam in a wild-type background produced low- and some intermediate-molecular-weight DpnI fragments, indicating that HDA-1–Dam localized to heterochromatin (Fig. 5A). Little DpnII digestion was
detected at the euchromatic pan-1 gene. Compared with a wild-type strain, HDA-1-Dam localization to heterochromatin was reduced in cdp-2 strains, providing further evidence that CDP-2 is required for proper targeting of HDA-1 (Fig. S4).

In principle, the essential role of HDA-1 in HCHC may or may not depend on HDAC activity. To distinguish between these possibilities, we generated an hda-1 construct with a point mutation causing an amino acid substitution of a residue critical for HDAC activity, HDA-1D263N (Fig. 5 B) (25), and with a 3xFLAG epitope tag at the C terminus of the protein. The construct was driven by the native hda-1 promoter and was inserted at the his-3 locus of an hda-1 mutant. Insertion of a wild-type control hda-1–FLAG construct restored nearly normal patterns of DNA methylation, indicating that the HDA-1–FLAG fusion was functional (Fig. 5C). In contrast, HDA-1D263N–FLAG failed to complement the methylation defects (Fig. 5C), even though it was expressed as well as wild-type HDA-1–FLAG (Fig. 5 D and E). Co-IP experiments verified that the mutation did not affect the stable formation of HCHC (Fig. 5D and E), implying that HDAC activity is required for the HCHC function. Taking these results together with the previous observation that mutants lacking CDP-2 show striking hyperacetylation of histones H3 and H4 at heterochromatic regions (22), we conclude that CDP-2 serves as a bridge between HP1 and HDA-1 to recruit HDAC activity to methylated H3K9 regions.

HP1 and CDP-2 Localize to Heterochromatin Independently of HDA-1 and CHAP. To investigate further the extent to which recruitment of HP1 and CDP-2 may depend on other members of the complex, we generated hda-1 and chap mutants expressing HP1–GFP or CDP-2–GFP and examined localization of the GFP-tagged proteins by microscopy. Because CDP-2 is destabilized in the absence of other components of the complex (Fig. S6 B–D), it was necessary to overexpress CDP-2 to test if its punctate localization depends on the other HCHC components. The nuclear foci that characterize normal HP1–GFP localization were lost when H3K9me3 was abrogated in a dim-3 mutant but were evident in cdp-2, hda-1, and chap mutants (Fig. S7A), consistent with the model that HP1 recruits CDP-2, HDA-1, and CHAP to chromatin marked with H3K9me3. Similarly, CDP-2 localization was unaltered in hda-1 and chap mutants but was dependent on HP1 and H3K9me3 (Fig. S7B). Furthermore, using the DamID assay, we verified that CDP-2 localization to heterochromatin depends on HP1 but not on HDA-1 and CHAP (Fig. S7C). These data suggest that CDP-2 is important for tethering HP1 to HDA-1/CHAP, as is consistent with our interaction map (Fig. 4B).

HP1 and CDP-2 both require the CHAP AT-hook motifs and the PxVxL-like motifs of CDP-2 for interaction with HDA-1, as verified by co-immunoprecipitation (Fig. 4, A and B). HDA-1 interacts with the first zinc-finger motif of CHAP. (C and D) Verification of the interaction of CDP-2 with HP1 and HDA-1 via the N-terminal PxVxL-like motifs of CDP-2 in vivo. Co-IP experiments were performed with anti-FLAG antibodies in strains with the indicated tagged proteins. Input and immunoprecipitation samples were fractionated and analyzed by Western blotting with antibodies against the indicated epitopes. The asterisk indicates nonspecific bands. Strains: N3808, N3836, 3440, 3443, 3445, and 3447.

The CHAP AT-Hook Motifs Specifically Bind AT-Rich DNA that Has Repeat-Induced DNA Methylation. To evaluate the possible role of the AT-hooks of CHAP, we generated a series of constructs with point mutations to change critical residues in these motifs and with a 3xHA epitope tag at the C terminus of the protein (CHAP-HA) (Fig. 7A). All constructs were driven by the native chap promoter and were inserted at the pan-2 locus of a chap-deletion strain. We confirmed that mutations in the AT-hook motifs (CHAPATh1, CHAPATh2, and CHAPATh1&2) did not affect the level of CHAP protein (Fig. S8B) and that insertion of a wild-type chap–HA construct into a chap-deletion strain restored nearly normal patterns of DNA methylation, indicating that the CHAP-HA fusion is functional (Fig. 7B). Strains expressing CHAPATh1 showed moderate restoration of DNA methylation, and strains expressing CHAPATh2 exhibited almost full restoration. However, strains bearing mutations in both AT-hook motifs (CHAPATh1&2) showed marked defects in DNA methylation (Fig. 7B). Co-IP experiments verified that the AT-hook mutations do not affect the stable formation of HCHC (Fig. S8C). We conclude that the CHAP AT-hook motifs are required for normal DNA methylation, perhaps through the AT-rich DNA-binding activity expected of such motifs (26).

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Fig. 5. HCHC function depends on the HDA-1 HDAC. (A) Sequence-dependent localization of HDA-1–Dam depends on CDP-2 and CHAP. Genomic DNA from a wild-type strain with (+) or without (−) HDA-1–Dam, as well as wild-type, cdp-2, and chap strains expressing HDA-1–Dam, were incubated with (+) or without (−) DpnII, which cuts adenine-methylated GATC sites. As a control for completely digested DNA, genomic DNA from the wild-type strain was incubated with the Smc-insensitive isoschizomer DpnI. Digested DNA was used for Southern hybridizations with probes for the methylated regions 8:A6, 8:G3, and cenVIIR as well as a euchromatic gene, pan-1. Strains: N3752, N3995, N4023, and N4082. (B) The position of the point mutation in the HDA-1 catalytic domain. Asparagine 263 is predicted to be essential for the HDAC activity (25). (C) The introduction of the hda-1 gene with the catalytic mutation does not complement DNA methylation defects in hda-1–null mutants. The experiment was carried out as described in Fig. 3B with the 8:A6 methylated region (17). Strains: N623, N1877, N3610, N3997, and N3998. (D and E) The HDA-1 catalytic mutation does not disrupt the HCHC complex. Co-IP experiments were performed with anti-FLAG antibodies in strains with (+) or without (−) the indicated tagged proteins. Input and immunoprecipitation samples were fractionated and analyzed by Western blotting with antibodies against the indicated epitopes. Strains: N3321, N4002, N4043, N3377, N4000, and N4699.

point mutations, we performed in vitro DNA-affinity purification with the recombinant CHAP N terminus (residues 1–274) containing the two AT-hook motifs and analyzed the purified DNA with high-throughput sequencing. To complement this approach, we also assessed the binding of CHAP in vivo with DamID sequencing using the CHAP–Dam strain. Together, these techniques gave us a detailed genomic view of the specific localization and binding of CHAP to AT-rich DNA that has repeat-induced point mutations, which is nearly coincident with methylated DNA regions (15). We previously demonstrated that HCHC function depends on the HDA-1 HDAC. (28) More than 30 years ago. However, it has become apparent that maintenance of methylation patterns reflects the product of a variety of processes involving a multitude of proteins. In addition to DNA methyltransferases and other enzymes that interact with DNA to convert or excise 5-methylcytosine (5mC) residues, chromatin remodelers and histone modification enzymes impact the distribution and intensity of DNA methylation (29). Indeed, in some organisms, such as N. crassa, DNA methylation is dependent on the methylation of a particular residue of histone H3, H3K9 (11). Other histone modifications, such as methylation of H3K4, phosphorylation of H3S10 (30, 31), and histone acetylation, also influence DNA methylation (32, 33). We previously demonstrated that HCHC mutants of N. crassa show increased acetylation of histone H3 and H4 at larger heterochromatin domains, such as centromeres, and speculated that the increased acetylation might provide an enhanced environment for the HP1–DIM-2 complex, leading to the increased DNA methylation observed in the large domains of constitutive heterochromatin in centromere regions. Our WGBS

Discussion
DNA methylation, a prototypical epigenetic mark, is widely thought to be stably maintained by a simple copying system at symmetric methylated sites, as proposed by Riggs (27) and Holliday and Pugh (28) more than 30 years ago. However, it has become apparent that maintenance of methylation patterns reflects the product of a variety of processes involving a multitude of proteins. In addition to DNA methyltransferases and other enzymes that interact with DNA to convert or excise 5-methylcytosine (5mC) residues, chromatin remodelers and histone modification enzymes impact the distribution and intensity of DNA methylation (29). Indeed, in some organisms, such as N. crassa, DNA methylation is dependent on the methylation of a particular residue of histone H3, H3K9 (11). Other histone modifications, such as methylation of H3K4, phosphorylation of H3S10 (30, 31), and histone acetylation, also influence DNA methylation (32, 33). We previously demonstrated that HCHC mutants of N. crassa show increased acetylation of histone H3 and H4 at larger heterochromatin domains, such as centromeres, and speculated that the increased acetylation might provide an enhanced environment for the HP1–DIM-2 complex, leading to the increased DNA methylation observed in the large domains of constitutive heterochromatin in centromere regions. Our WGBS
analyses on a wild-type strain confirmed that shorter regions tend to be more methylated than longer regions (Fig. 2A), whereas HCHC mutants show hypomethylation of shorter regions and hypermethylation of longer regions (Fig. 2A and B). The current study also demonstrated that the AT-hook motifs of CHAP are important for proper DNA methylation and bind specifically to AT-rich DNA that has repeat-induced point mutations (Fig. 8), which is particularly prevalent at centromere regions. This finding raises the possibility that CHAP binding contributes to stronger recruitment of HCHC at centromeres, at the expense of the HP1–DIM-2 complex, leading to the characteristic low levels of DNA methylation in these regions. It is interesting that, in contrast to the importance of DNA methylation in silencing short heterochromatic regions, DNA methylation is unnecessary for silencing at centromere regions (22).

The HCHC complex possesses two CD proteins, HP1 and CDP-2, which one might imagine could operate semiredundantly. Consistent with this possibility, we found that although the CD of CDP-2 binds efficiently to methylated H3K9 in vitro (22), this domain is not required for normal DNA methylation and centromere silencing in vivo (Fig. 3B and Fig. S2 B and C). We therefore considered the possibility that the CDP-2 CD in the HCHC complex might mediate the association of this complex with methylated H3K9 in the absence of HP1 binding. However, the CDP-2 CD mutants did not show additional DNA methylation defects in the HP1 CD–mutant background (Fig. 3E), suggesting that the CDP-2 CD does not work redundantly with the HP1 CD in HCHC. Instead, our findings suggest that CDP-2 serves as a bridge between HP1 and HDA-1, ensuring the proper recruitment of the HDA-1 HDAC domain to chromatin (Fig. 9B). We show that CDP-2, like DIM-2, interacts directly with the HP1 chromoshadow domain through the PxVxL-like motifs near the N terminus (Fig. 4B and C). This observation is consistent with our previous observation that HP1 forms physically and functionally distinct complexes with DIM-2 and CDP-2 (22).

We demonstrated that in the absence of the HP1 CD, N. crassa still has residual DNA methylation in the regions with repeat-induced point mutations that depend on HDA-1. This surprising residual DNA methylation is dependent on CHAP (Fig. 6), which apparently serves as an additional means to recruit HCHC that is independent of the chromodomains (Fig. 9D). Therefore, we propose that dual chromatin recognition of heterochromatin by the HP1 CD and by the CHAP AT-hook motifs is responsible for HCHC function (Fig. 9A). Curiously, we still observed DNA methylation at the 8:F10 region is unchanged in mutants lacking the HP1 CD or CHAP show the residual DNA methylation at the 8:A6, 8:G3, and 2:B3, whereas the double mutants show complete loss of DNA methylation. DNA methylation at the 8:F10 region is unchanged in mutants lacking the HP1 CD and/or CHAP. Strains: N3753, N5580, N6166, N6390, N6392, and N6391.

**Fig. 6.** CHAP is essential for the residual DNA methylation in the HP1 CD mutant. Southern blot analysis was carried out as in Fig. 3B. The three upper panels are the hypomethylated 8:A6, 8:G3, and 2:B3 regions, and the bottom panel is the intact methylated 8:F10 region in hda-1-null mutants. Mutants lacking the HP1 CD or CHAP show the residual DNA methylation at the 8:A6, 8:G3, and 2:B3, whereas the double mutants show complete loss of DNA methylation. DNA methylation at the 8:F10 region is unchanged in mutants lacking the HP1 CD and/or CHAP. Strains: N3753, N5580, N6166, N6390, N6392, and N6391.
methyltransferase in double mutants lacking the HP1 CD and CHAP at the region 8:F10, which has repeat-induced point mutations (Fig. 6), raising the possibility that another element of HP1 recognizes a heterochromatic signal. In mammals and fission yeasts, HP1 has been shown to bind to RNA through the hinge region in addition to binding methylated H3K9 through the CD (34, 35). Although the RNAi pathway is not involved in heterochromatin formation in N. crassa (36), bivalent recognition via the CD and hinge region of HP1 seems possible.

Although N. crassa has a relatively simple DNA methylation pathway centered on H3K9 methylation serving as a signal for the direct recruitment of the HP1–DIM-2 complex (13, 14), reinforcing loops involving H3K9me3, HP1, and DNA methylation occur. Recent studies using N. crassa and Arabidopsis uncovered mutants that fail to modulate these reinforcing loops properly (37, 38). The mutants exhibit abnormal silencing of essential genes by aberrant DNA methylation and H3K9 methylation, resulting in growth defects. In N. crassa, aberrant H3K9me3 depends on DNA methylation, revealing the existence of feedback pathways from DNA methylation to H3K9me3 (37). In addition, our WGBS analyses revealed that HDA-1 and CHAP are required for the spreading of DNA methylation (Fig. 2D), presumably through their binding of HDAC and AT-rich DNA that has repeat-induced point mutations. In summary, we describe multifaceted interrelationships among AT-rich DNA that has repeat-induced point mutations, H3K9me3, HP1, histone deacetylation, and DNA methylation that together result in the observed establishment and maintenance of heterochromatic domains.

The N. crassa HHC complex shares features with the Schizosaccharomyces pombe HDAC complex SHREC (25, 39), which also functions in centromeric silencing. Although there are obvious differences between the HDAC complexes in fission yeast and N. crassa (e.g., N. crassa HHC does not contain a homolog of the chromatin remodeler Mit1), it would be interesting to know if mammals use a similar mechanism to control proper heterochromatin domains, especially at AT-rich pericentromeric heterochromatin.

Materials and Methods

N. crassa Strains and Molecular Analyses. All N. crassa strains and primers used in this study are listed in Tables S1 and S2, respectively. Strains were grown, crossed, and maintained according to standard procedures (40). N. crassa strains were built according to methods described previously (41). Detailed methods for strain building, including plasmid and primers used are included in SI Materials and Methods. DNA isolation, Southern blotting, Western blotting, co-IP, and fluorescence microscopy were performed as previously described (14). The following antibodies were used: anti-FLAG (Sigma, F3165; MBL, M185-3), anti-HA (University of Oregon monoclonal), anti-CDP-2, anti-HDA-1, and anti-tubulin (Sigma, T6199). Specific HP1, CDP-2, HDA-1, and CHAP mutations were made with a QuikChange site-directed mutagenesis kit (Stratagene) and a PCR-based mutagenesis with the In-Fusion HD cloning system (Takara).
Assessment of Chromosome Bridges. The frequency of chromosome bridges was quantified using with GFP-tagged histone H2A (20). Conidia were plated on a thin layer of Vogel’s medium solidified with 2% agar and supplemented with required nutrients and were grown at 32 °C overnight. A square of the culture was placed on a slide and covered with a drop of Vogel’s medium and a coverslip. Hyphae observed using a 100× oil-immersion objective in a Zeiss Axioplan 2 fluorescence microscope with differential interference contrast (DIC) showed cytoplasmic streaming. Two methods were used to count chromosome bridges that were visualized with a GFP filter. (i) For tips with at least four nuclei, those having a chromosome bridge were scored as positive. (ii) In other hyphae (in which the tips were not obvious), the number of bridges was recorded relative to the total number of nuclei observed. To combine the results from the two counting schemes, the number of tips multiplied by 4 was used as the number of nuclei for the first counting method. Results were expressed as the percentage of nuclei showing bridges relative to the total number of nuclei. For each nucleus there are two possible outcomes: having a bridge or not having a bridge. Thus, we used the statistical test for a binomial distribution. The 95% confidence intervals were calculated for the binomial parameter \( p \), the probability of a bridge in a given strain, using the formula \( \sqrt{p(1-p)/n} \) where \( n \) is the total number of nuclei observed.

WGBS. WGBS was performed and reads were mapped as previously described (42). Sequencing reads can be downloaded from the National Center for Biotechnology (NCBI) database (accession no. GSE81129). Normally methylated regions with a minimum size of 200 bp were determined using the RSEG software package (smithlabresearch.org/software/rseg/). To display the bisulfite sequencing data, the average 5mC level was determined for specified step-wise window sizes across the genome using the MethPipe program (smithlabresearch.org/software/methpipe) (43). The resulting file was renamed with an .igv file extension to allow display on the Integrated Genome Viewer (software.broadinstitute.org/software/igv) (44). Similarly, the MethPipe (ROI function) was used to calculate the average SmC level over the normally methylated regions found in the wild-type strain (N3752) as determined using RSEG software and sequences immediately flanking these regions. The CRI was calculated for 500-bp windows across the N. crassa genome using a custom Perl script (15).

CHAP–DamID Sequencing. Whole-genome DamID sequencing was performed using a procedure adapted from ref. 46. Briefly, genomic DNA from the Dam-tagged CHAP strain was digested with DpnI. Digested DNA was ligated to adapters and amplified using a biotin-tagged primer. The amplified DNA was fragmented by sonication to 100- to 500-bp products and purified using streptavidin-conjugated beads (Sigma). Bound DNA was eluted using a DpnII digestion. Purified DNA was prepared for sequencing using the Illumina TruSeq ChIP Sample Preparation Kit. Sequence alignments were performed as previously described (47), except that the reads were mapped to the N. crassa OR74A (NC12) genome (N. crassa Sequencing Project, Broad Institute of Harvard and MIT; www.broadinstitute.org/), and read densities then were averaged over 25-bp windows to generate all tiled data files. Sequencing reads can be downloaded from the NCBI database (accession no. GSE81129).
Construction of HA-Tagged CHAP Fusion Constructs Expressed at the pan-2 Locus. We amplified a fragment of HA-tagged chaperone gene with its native promoter by PCR with primers 2090 and 2497 from the genomic DNA of a strain expressing CHAP–HA from its native locus (created using the knock-in system described above). The PCR products were digested with NotI and XhoI, inserted into the pan-2 targeting vector pRATT42b (the gift of R. Aramayo, Texas A&M University), linearized, and inserted at the pan-2::hph::tk locus of the chap-null mutant (N3642).

Generation of Recombinant CHAP Proteins and Gel Mobility Shift Assays. The chaperorin ORF (amino acids 1–274) was amplified with primers 2011 and 3069 and inserted between the SacI and BamHI sites of pMALc2 (New England Biolabs). The plasmids were transfected into E. coli strain BL21, and recombinant proteins were purified as described by the manufacturer of pMALc2. Recombinant maltose binding protein (MBP)-CHAP1–274 protein was incubated for 30 min at room temperature in a 20-µL volume of EMSA binding buffer [20 mM Hepes (pH 7.9), 50 mM KCl, 4 mM MgCl₂, 25 µM ZnCl₂, and 1 mM DTT], 1 µg of BSA, and a radiolabeled DNA probe. A 100-pM DNA probe was used for Kₐ determination. Double-stranded DNA probes were produced using PCR primers (probe 1: primers 1 and 2020; probe 2: primers 3 and 3019). The probes were separated by electrophoresis in 1% agarose gel and then blotted onto a nitrocellulose membrane. After incubation, the membrane was dried and hybridized against a radiolabeled probe.

Mini-Protein TGX gels (Bio-Rad); after electrophoresis, gels were dried and autoradiographed.

DNA-Protein Affinity Purification. DNA affinity purification using recombinant MBP-CHAP was performed using a protocol adapted from ref. 48. Amyllose resin (New England Biolabs) containing immobilized MBP-CHAP was incubated with sonicated wild-type genomic DNA (∼250-bp fragments) in binding buffer [20 mM Hepes (pH 7.5), 80 mM NaCl, 37.5 mM imidazole, 0.7 mM MgCl₂, 0.35 mM EDTA, 0.7 mM DTT, and 17.8% glycerol] for 2 h at 4 °C. Beads were washed six times with 1 mL of wash buffer [10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, and 4% glycerol]. Following washes, DNA was eluted with TES [20 mM Tris (pH 8.0), 10 mM EDTA, and 1% SDS] by heating for 10 min at 65 °C. Eluted samples were treated with proteinase K, and DNA was purified with MinElute columns (Qiagen). Purified DNA was prepared for high-throughput sequencing using the Truseq ChiP Sample Preparation Kit (Illumina).

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