Histone H1 Limits DNA Methylation in Neurospora crassa

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ABSTRACT Histone H1 variants, known as linker histones, are essential chromatin components in higher eukaryotes, yet compared to the core histones relatively little is known about their in vivo functions. The filamentous fungus Neurospora crassa encodes a single H1 protein that is not essential for viability. To investigate the role of N. crassa H1, we constructed a functional FLAG-tagged H1 fusion protein and performed genomic and molecular analyses. Cell fractionation experiments showed that H1-3XFLAG is a chromatin binding protein. Chromatin-immunoprecipitation combined with sequencing (ChIP-seq) revealed that H1-3XFLAG is globally enriched throughout the genome with a subtle preference for promoters of expressed genes. In mammals, the stoichiometry of H1 impacts nucleosome repeat length. To determine if H1 impacts nucleosome occupancy or nucleosome positioning in N. crassa, we performed micrococcal nuclease digestion in the wild-type and the ΔhH1 strain followed by sequencing (MNase-seq). Deletion of hH1 did not significantly impact nucleosome positioning or nucleosome occupancy. Analysis of DNA methylation by whole-genome bisulfite sequencing (MethylC-seq) revealed a modest but global increase in DNA methylation in the ΔhH1 mutant. Together, these data suggest that H1 acts as a nonspecific chromatin binding protein that can limit accessibility of the DNA methylation machinery in N. crassa.

In eukaryotes, packaging of genomic DNA into chromatin is essential for genome function. The most basic unit of the chromatin fiber is the nucleosome core particle (NCP), made up of ~146 bp of DNA wrapped around a core of histones H3, H4, H2A, and H2B (Kornberg and Thomas 1974; Oli三 and Oli三 1974; Luger et al. 1997). In addition to the core histones, many organisms encode one or more H1 proteins, also known as linker histones. H1 proteins are evolutionarily unrelated to the core histones and are characterized by a central winged helix domain, or globular domain, flanked by unstructured N- and C-termini (Cerf et al. 1993; Ramakrishnan et al. 1993; Kasinsky et al. 2001). Early studies showed that animal H1 proteins bind outside of the NCP (Baldwin et al. 1975; Shaw et al. 1976) and can protect an additional 20 bp of DNA from nuclease digestion (Whitlock and Simpson 1976; Noll and Kornberg 1977). Subsequent studies revealed that H1 binds near the NCP dyad axis and can interact with DNA as it enters and exits the NCP (recently reviewed in Bednar et al. 2016). Although the interactions between H1 and the NCP have been extensively investigated, H1’s roles in the cell remain poorly understood.

In vivo studies of mammalian H1 are complicated by the existence of 11 H1 variants that appear to be partially redundant (Pan and Fan 2015). Deletion of single H1 variants failed to produce significant phenotypes in mice (Fan et al. 2001), but mice lacking three H1 variants are inviable (Fan et al. 2003) and triple-knockout embryonic stem cells (ESCs) are unable to differentiate (Zhang et al. 2012a). These and other data suggest that animal H1 variants cooperate to perform critical functions, influencing gene regulation (Fan et al. 2005; Li et al. 2012b; Zhang et al. 2012b), establishment, and/or maintenance of chromatin modification patterns (Li et al. 2012b; Zhang et al. 2012a; Yang et al. 2013; Lu et al. 2013) and formation of higher order chromatin structures (Fan et al. 2005; Geeven et al. 2015).

KEYWORDS chromatin histone H1 nucleosome positioning DNA methylation
Less is known about the functions of H1 in other groups of organisms, but genetic studies have been carried out in a handful of microbial model systems. H1 is not essential for viability in the single-celled Saccharomyces cerevisiae (Patterson et al. 1998) or Tetrahymena thermophila (Shen et al. 1995). Similarly, H1-deficient mutants are viable in several filamentous fungi including Neurospora crassa (Folco et al. 2003), Aspergillus nidulans (Ramón et al. 2000), and Ascomobolus immersus (Barra et al. 2000). The yeast H1 homolog Hho1p suppresses homologous recombination (Downs et al. 2003; Li et al. 2008), impacts ribosomal RNA processing (Levy et al. 2008), and influences chromatin compaction during stationary phase (Schäfer et al. 2008). In T. thermophila, H1 is required for normal chromatin compaction in macronuclei and influences expression of a small number of genes (Shen et al. 1995; Shen and Gorovsky 1996). It is important to note that both yeast Hho1p and T. thermophila H1 have atypical protein structures. The yeast protein contains two globular domains, whereas the T. thermophila protein lacks a globular domain altogether. Thus, it is not clear if these proteins are functionally analogous to H1 in other organisms.

The filamentous fungi N. crassa, A. nidulans, and A. immersus encode H1 proteins with a canonical tripartite structure, raising the possibility that these genetic systems can be used to gain insights into H1 function in plants and animals. In N. crassa, an hH1-deficient strain displayed reduced growth and H1 was required for repression of the efp gene in the presence of ethanol (Folco et al. 2003). In A. immersus, H1 gene silencing led to increased nucleosome accessibility and increased DNA methylation (Barra et al. 2000). In contrast, deletion of hhoA in A. nidulans failed to produce a phenotype (Ramón et al. 2000). In general, the functions of H1 in filamentous fungi remain poorly understood. Moreover, it is not clear if H1 plays similar roles in fungal and animal cells. In the present study, we utilized molecular and genomic approaches to investigate the functions of H1 in the model fungus N. crassa. We confirmed that N. crassa H1 is a chromatin component in vivo, and we found that H1 is not a major determinant of nucleosome positioning, nucleosome repeat length, or nucleosome occupancy. We report that an H1 fusion protein exhibits enhanced enrichment at nucleosome-free regions and is depleted from coding sequences of expressed genes in a ChIP-seq assay. We also show that loss of H1 causes a global increase in DNA methylation.

MATERIALS AND METHODS

Strains, growth media, and molecular analyses

All Neurospora strains used in this study are listed in Table 1. Knockout strains of hH1 were generated by the N. crassa gene knockout consortium (Colot et al. 2006) and obtained from the Fungal Genetics Stock Center (McCloskey et al. 2010). Neurospora cultures were grown at 32° in Vogel’s minimal medium (VMM) + 1.5% sucrose (Davis et al. 1970). Crosses were performed on modified synthetic medium at 25° (Davis et al. 1970). For mating assays, N. crassa conidia were plated on VMM with 2.0% sorbose, 0.5% fructose, and 0.5% glucose. When relevant, plates included 200 μg/ml hygromycin or 400 μg/ml basta (Pall et al. 2003) using the Burrows-Wheeler Aligner (BWA version 0.7.10) according to the manufacturer’s instructions with the following modification. Library amplification was performed using only four cycles of PCR to reduce biased enrichment of GC-rich DNA (Ji et al. 2014). Libraries were sequenced at the University of Georgia Genomics Facility on an Illumina NextSeq 500 instrument. Reads were aligned to version 12 of the N. crassa genome (Refseq Accession # GCF_000182925.2; Galagan et al. 2003) using the Burrows-Wheeler Aligner (BWA version 0.7.10) (Li and Durbin 2009). To determine if H1-3XFLAG was enriched over background, coverage was normalized to mitochondrial DNA as follows. We used BEDtools (version 2.25.0) ‘coverage’ to calculate read coverage for 1000 bp windows across the genome (Quinlan and Hall 2010). We then used BEDtools ‘map’ to calculate the median coverage for mitochondrial DNA. The coverage for each 1000 bp window was then divided by the median coverage for mitochondrial DNA. As a positive control, data from a previously published ChIP experiment for methylated lysine-9 of H3 was analyzed (Accession #SRX550120; Sasaki et al. 2014).

The Hypergeometric Optimization of the Motif EnRichment (HOMER version 4.7.2) software package (Heinz et al. 2010) was used to generate metatplots and heatmaps of enrichment data (annotatePeaks.pl module; using the -hist and -ghist option, respectively). We first created a custom HOMER genome annotation for Neurospora using a fasta file and a GTF file (Supplemental Material, File S1) containing the version 12 genome assemblies and annotations, respectively (Galagan et al. 2003). All plots were centered on transcriptional start sites or transcriptional termination sites, and a window size of 10 bp was specified for all histograms (-hist 10). HOMER was also used to construct metatplots of expression-ranked gene groups using the -list option. Genes were assigned into expression-ranked groups by expression level determined by RNA-seq (see below). RPKM values for each quintile group were: Q1 (12927.9–36.8); Q2 (36.8–13.8); Q3 (13.8–4.9); Q4 (4.9–0.33); Q5 (0.33–0). Thus, most genes in expression group 5 were silent or rarely expressed.
Table 1 Strains used in this study

| Lab Strain # | Description | Source |
|-------------|-------------|--------|
| S1          | FGSC #4200 wild-type mat a | (McCluskey et al. 2010) |
| S2          | FGSC #2489 wild-type mat A | (McCluskey et al. 2010) |
| S240        | hH1::xflg-Hph+ mus-S2::Basta | (This study) |
| FGSC #12224 | ΔhH1::Hph+ mat A | (Colot et al. 2006) |
| S123        | Δdim-2::Hph+ mat A | (Kouzminova and Selker 2001) |
| S94         | hH1RFP2 | (Folco et al. 2003) |

MNase digestion: For micrococcal nuclease (MNase) experiments, 5 x 10^6 conidia/ml were inoculated into 50 ml of VMM and incubated at 32° for 5 hr. The cell suspension was transferred to a 50 ml conical flask and centrifuged at 1000 x g for 5 min to pellet germinated conidia. Cell pellets were washed with 10 ml Phosphate Buffered Saline (PBS) (Sambrook et al. 1989) and then resuspended in 10 ml of PBS containing 1% formaldehyde. The cell suspension was transferred to a 125 ml flask and incubated with gentle shaking for 30 min at room temperature before the cross-linking agent was quenched by addition of 500 ml 2.5 M glycine. The cell suspension was transferred to a 50 ml conical tube and germinating conidia were pelleted by centrifugation for 5 min at 1000 x g. Cells were washed once in 40 ml of PBS and resuspended in 1 ml of ice-cold PBS. Cells were pelleted by centrifugation for 5 min at 5000 x g, and each cell pellet was resuspended in NPS buffer with calcium chloride [50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 1% Triton X-100, 0.1% deoxycholate, 0.5 mM spermidine, 1 mM PMSF plus Roche EDTA-free protease inhibitor tablets (catalog # 05892791001)]. Cells were then lysed by gentle sonication using a Heat Systems, Ultrasonics W-380 sonicator with micro tip (Output 2.0, Duty Cycle 80%; 30 1 sec pulses). The chromatin fraction was pelleted by centrifugation for 5 min at 14,000 x g. The supernatent was discarded and each pellet was resuspended in 1 ml of NPS buffer and transferred to a 15 ml conical tube. NPS buffer with calcium chloride was added to raise the volume to 6 ml and the chromatin sample was mixed by pipetting. Then, 700 µl aliquots were transferred to 1.5 ml tubes and 2 units of micrococcal nuclease (cat # 2910A; Clontech) were added to each tube. Individual samples were incubated at 37° for 5, 10, 20, 40, or 60 min. MNase digestions were stopped by addition of 15 µl 0.5M EDTA and 25 µl 4M NaCl, and samples were incubated overnight at 65° to reverse cross-links. A total of 6 µl RNase A (10 mg/ml; Fisher Scientific, cat # BP2529250) was added and samples were incubated for 2 hr at 50°. A total of 6 µl of 10% SDS and 10 µl Proteinase K (10 mg/ml; Fisher Scientific, cat # BP1700) was then added and samples were incubated for 2 hr at 65°. The digested DNA was isolated by phenol-chloroform extraction and precipitated overnight at −20° in ethanol and sodium acetate (Sambrook et al. 1989). Digested DNA was resolved by gel electrophoresis to confirm that digestion was successful.

MNase-seq: We constructed sequencing libraries from mononucleosomes generated by 20 or 60 min MNase digestion. We first performed a gel extraction (Qiagen, cat #28706) of mononucleosomal DNA (~150 bp) and constructed libraries using an Illumina TruSeq Sample preparation kit (Illumina) according to the manufacturer’s instructions. A total of 50 bp paired-end sequencing reads were generated on an Illumina HiSeq 2500 instrument at the Oregon State University genomics core facility. Due to a technical problem during the sequencing run, only 44 bp of sequence were obtained for the read 2 sequence. Sequences were mapped to the N. crassa version 12 genome assembly (Galagan et al. 2003) using bowtie2 (version 2.2.3) (Langmead and Salzberg 2012). To analyze the size distributions in the wild-type and the hH1 strain, the Picard software package (http://broadinstitute.github.io/picard) was used to remove duplicate reads and determine insert size metrics (using CollectInsertSizeMetrics). HOMER was used to create metaplots of MNase data as described above. In all cases, a window size of 10 bp was used (-hist 10). Metaplots depict only plus strand reads (using the ‘+strand’ option) and thus peaks indicate the left edge of nucleosomes.

RNA-seq: For RNA-seq experiments, 5 x 10^6 conidia/ml were inoculated into 50 ml of VMM containing 2% glucose and grown for 5 hr at 32°. RNA isolation was performed as described (Bell-Pedersen et al. 1996; Schwerdtfeger and Linden 2001), and strand-specific RNA-seq libraries were prepared from 5 µg total RNA. Ribosomal RNAs were depleted using the yeast Ribo-zero kit (cat # MRZ1324 Epicentre) and RNA libraries were generated with the Illumina Stranded RNA-seq kit (cat # RS-122-2101). Reads were aligned to version 12 of the N. crassa genome sequence using TopHat (Trapnell et al. 2009) and expression levels (RPKM) were determined using cufflinks (Trapnell et al. 2012).

MethylC-seq: For DNA methylation analysis, conidia were inoculated into 50 ml of VMM and cultures were grown for 48 hr at 32°. Genomic DNA was isolated using described procedures (Pomraning et al. 2009). MethylC-seq libraries were prepared as previously described (Urich et al. 2015). Illumina sequencing was performed at the University of Georgia Genomics Facility using an Illumina NextSeq 500 instrument. Sequencing reads were trimmed for adapters, preprocessed to remove low quality reads, and aligned to the N. crassa version 12 genome assembly (Galagan et al. 2003) as described in Schmitz et al. (2013). Mitochondrial DNA sequence (which is fully unmethylated) was used as a control to calculate the sodium bisulfite reaction nonconversion rate of unmodified cytosines. Only cytosine sites with a minimum coverage (set as 3) were allowed for subsequent analysis. Binomial test coupled with Benjamini-Hochberg correction was adopted to determine the methylation status of each cytosine. Identification of DMRs (Differentially Methylated Regions) was performed as described (Schultz et al. 2015). Methylated regions in the wild-type were defined previously (Basenko et al. 2016). For metaplots, both upstream and downstream regions were divided into 20 bins each of 50 bp in length for a total 1 kb in each direction. Methylated regions were separated every 5%, for a total of 20 bins. Weighted methylation levels were computed for each bin as described previously (Schultz et al. 2012).

Data availability
All strains are listed in Table 1 and available upon request or from the Fungal Genetics Stock Center (Manhattan, KS). All sequencing data have been deposited into the NCBI SRA/GEO databases. ChiP-seq, MNase-Seq, and RNA-seq data generated during this study have been deposited under accession #GSE78157. Control data from a previously published ChiP-seq experiment for methylation of H3 lysine-9 was deposited under accession #SRX550120 (Sasaki et al. 2014). MethylC-seq
data are deposited under accession #GSE76982 (this study) and #GSE70518 (Basenko et al. 2016).

RESULTS

Construction of an H1-3XFLAG fusion protein

To investigate the role of H1 in N. crassa cells, we constructed an epitope-tagged version of the protein by introducing coding sequence for a 3X-FLAG tag at the 3' end of the native hH1 locus (Figure 1A). Primary transformants were crossed to obtain a homokaryon that was analyzed further. To confirm that the H1-3XFLAG fusion protein is functional, we first compared the growth rate of the hH1::hH1-3xflag-Hph+ strain to the wild-type and to an hH1 deletion strain obtained from the Neurospora gene knockout consortium (Colot et al. 2006). The ΔhH1 strain displayed a reduced growth rate, as reported previously for an H1 loss-of-function allele generated by repeat-induced point mutation (Folco et al. 2003). The hH1::hH1-3xflag-Hph+ grew similar to the wild-type (Figure 1, B and C). We also asked if the H1-3XFLAG protein associates with chromatin. We isolated soluble and chromatin-containing fractions (see Materials and Methods) and performed western blot analyses using anti-FLAG and anti-H3 antibodies. Western blots probed with an anti-FLAG antibody revealed a single band. The apparent molecular weight was larger than expected based on amino acid sequence prediction, but the apparent size was consistent with previous analysis of N. crassa purified by extraction with perchloric acid (Folco et al. 2003). We detected H1-3XFLAG in both soluble and chromatin fractions with higher levels of H1-3XFLAG observed in the chromatin fraction (Figure 1D). As expected, H3 was exclusively detected in the chromatin fraction. Together, these data demonstrate that the H1-3XFLAG construct is functional and that N. crassa H1 is a component of chromatin.

H1 is moderately enriched in promoters and depleted from coding sequences of actively expressed genes

To determine the genome-wide distribution of H1-3XFLAG, we performed chromatin-immunoprecipitation followed by sequencing (ChIP-seq). Inspection of the H1-3XFLAG enrichment data in a genome browser revealed a relatively uniform distribution for all seven chromosomes. Given that high levels of H1-3XFLAG were detected in the chromatin fraction by western blotting, we reasoned that the uniform enrichment pattern observed for H1-3XFLAG might reflect global binding of H1 across the genome. To determine if this was the case, we normalized read counts obtained for each 1000 bp window in the nuclear genome to the median read count obtained for all 1000 bp windows covering the mitochondrial genome. This allowed us to calculate enrichment over background because mitochondrial DNA should not be enriched by immunoprecipitation of a nuclear protein. We plotted normalized enrichment data for all 1000 bp windows on Linkage Group VII (Figure 2). ChIP-seq experiments using anti-FLAG antibodies led to global enrichment of chromosomal DNA from the hH1::hH1-3xflag-Hph+ strain but not from a wild-type negative control strain. As a positive control, we normalized read counts

Figure 1 H1-3XFLAG is functional and binds to chromatin. (A) A cartoon illustrating the strategy for introducing a 3x-flag sequence into the 3’ end of the native hH1 locus by homologous recombination is shown. (B) The linear growth rate of the indicated strains was measured using ‘race tubes’ for three replicates of each strain. The direction of growth is left to right. (C) Quantification of the linear growth rate data from panel B as distance (y-axis; cm) vs. time (x-axis; hr). The three lines shown for each genotype represent three independent race tubes. (D) The soluble (Sol) and the chromatin fractions (Chr) were isolated from wild-type and the hH1::hH1-3xflag-Hph+ strain, and both fractions were analyzed by western blotting using anti-FLAG and anti-H3 antibodies, as indicated. MW, positions and sizes in kDa of a prestained protein ladder; WT, wild-type.
obtained in a previously published ChIP-seq experiment performed with antibodies to H3 methylated at lysine-9 (Sasaki et al. 2014). Results for other chromosomes were comparable. This confirmed that normalization to mitochondrial DNA is an effective method for quantifying enrichment over background. Moreover, these data demonstrate that H1 is a general chromatin architectural protein in *N. crassa*.

We did not detect prominent peaks that are typical of transcription factors or previously analyzed histone modifications such as H3K4me2 or H3K9me3 (Lewis et al. 2009). However, we did detect a subtle enrichment of H1 in the promoters of many genes along with a corresponding depletion of H1-3XFLAG within many coding sequences. To determine if this pattern occurred broadly across the *N. crassa* chromatin landscape, we created metaplots to analyze the average H1 distribution across the transcriptional start sites (TSS) or transcriptional termination sites (TTS) of all *N. crassa* genes (Figure 3A).

On average, H1-3XFLAG was enriched upstream of the TSS and was depleted from gene bodies. Similar enrichment patterns were observed for both biological replicates, but not in a negative control experiment in which we used anti-FLAG antibodies to perform ChIP-seq in a wild-type strain (no FLAG-tagged protein). A slight depletion of reads just upstream of the TSS was observed in negative control experiments. Given that these regions are typically depleted for nucleosomes (Sancar et al. 2015), this result may indicate that the FLAG antibody exhibits weak background binding to nucleosomal DNA. We next asked if H1-3XFLAG enrichment was correlated with the level of transcription. We binned *Neurospora* genes into five groups based on expression level and constructed metaplots to visualize the average H1-3XFLAG distribution pattern for each group (see Materials and Methods). The level of enrichment in gene promoters was positively correlated with the level of transcription (Figure 3B). Similarly, depletion of H1-3XFLAG from coding regions was correlated with the level of transcription. To explore this further, we performed ChIP-seq for RNA polymerase II and constructed heatmaps of H1-3XFLAG and RNA polymerase II occupancy across all Neurospora genes ordered by expression level (Figure 3, C and D). These data further support the idea that H1-3XFLAG enrichment is highest in the promoters and lowest in the coding sequences of highly expressed genes. We did not detect significant enrichment or depletion of H1-3XFLAG in heterochromatin domains.

**H1 is not a major determinant of nucleosome positioning in *N. crassa***

Given that H1-3XFLAG displayed a subtle preference for promoters of actively expressed genes, we hypothesized that H1 may impact chromatin structure at promoters. Like many eukaryotes, *N. crassa* promoters are characterized by nucleosome-free regions (NFRs) upstream of the TSS followed by well positioned “+1” nucleosomes (Sancar et al. 2015). To determine if H1 is important for the establishment of this characteristic promoter structure, and to determine if *N. crassa* H1 is important for nucleosome occupancy or nucleosome positioning at specific sites in the genome, we performed micrococcal nuclease digestion followed by sequencing (MNase-seq). Nuclei were incubated with MNase for increasing times (up to 60 min) before the DNA from each digestion reaction was purified and resolved on an agarose gel. Wild-type and ΔhH1 showed similar global MNase sensitivity across multiple experiments (representative gels are shown in Figure 4, A and B), with slightly enhanced MNase accessibility observed in the H1-deficient strain treated with enzyme for 5 or 10 min. This subtle increase in MNase accessibility in the ΔhH1 is in agreement with previously published work (Folco et al. 2003). We next performed gel extractions to isolate the DNA band corresponding to mononucleosomes (~150 bp) from samples digested with MNase for 20 or 60 min and constructed Illumina sequencing libraries. For each digestion time, libraries were prepared from two independent biological replicates (four samples in total for each strain) and paired-end sequencing was performed. We observed no differences in the average length of the mononucleosomal fragments in wild-type and ΔhH1 (Table 2). As expected, longer digestion times produced shorter DNA fragments (compare 20 min to 60 min digestion times).

We next asked if H1 impacted the occupancy and/or positioning of nucleosomes in promoters or gene coding sequences. We created metaplots to analyze the average nucleosome distribution across all *N. crassa* genes (Figure 4C). In this case, only plus strand reads were plotted to generate peaks corresponding to the edge of each nucleosome. The average gene profile revealed a characteristic nucleosome-free region upstream of the TSS, as reported previously for *N. crassa* (Sancar et al. 2015). The average nucleosome position profiles were similar in the wild-type and ΔhH1. Because H1 was most enriched in the promoters of highly expressed genes, we next plotted average MNase-seq enrichment profiles for genes grouped by expression level. Although we detected a subtle increase in the size of the NFR from the most expressed genes in the two 60 min digestion samples, this difference was not apparent in the samples subjected to a 20 min MNase digestion (Figure S1). The difference was also not apparent in a third independent replicate subjected to single-end Illumina sequencing (Figure S1). Thus, we conclude that H1 is not a major determinant of nucleosome positioning or occupancy in *Neurospora*. Because it appeared that enrichment of H1-3XFLAG was inversely correlated with nucleosome occupancy, we plotted plus strand reads from replicate one of the H1-3XFLAG ChIP-seq experiment described above to allow direct comparison of nucleosome occupancy and H1-3XFLAG.
**Δhh1 exhibits increased DNA methylation in N. crassa heterochromatin domains**

H1 impacts DNA methylation levels in animals (Fan et al. 2005; Zhang et al. 2012a), plants (Wierzbiicki and Jerzmanowski 2005; Zemach et al. 2013; Rea et al. 2012), and the fungus *A. immersus* (Barra et al. 2000). It was previously reported that H1 did not impact DNA methylation in *N. crassa* (Folco et al. 2003); however, Folco and colleagues analyzed only a single methylated region. It remained possible that H1 impacts DNA methylation in a region-specific manner or, alternatively, that H1 has a subtle impact on DNA methylation that may have been overlooked. We performed MethylC-seq to analyze DNA methylation across the entire genome at single base pair resolution. Genomic DNA was isolated from two replicates each of a wild-type *mat A* strain, the Δhh1 mutant, and a negative control Δdim-2 strain, which lacks DNA methylation altogether (Kouzminova and Selker 2001). All strains were grown simultaneously, but the wild-type and Δdim-2 data were published previously as part of another study (Basenko et al. 2016).

To determine if H1 impacts the level of DNA methylation in *N. crassa*, we first plotted DNA methylation levels across Linkage Group VII, a 4 Mb chromosome corresponding to ~10% of the genome. The overall pattern of DNA methylation was similar in the wild-type and Δhh1 strains. However, we noted that DNA methylation levels were higher in the Δhh1 mutant at most regions along the chromosome (Figure 5A). We next constructed metaplots to quantify the average methylation level for all genomic regions that are normally methylated in the wild-type *mat A* strain (see Materials and Methods). Both Δhh1 replicates displayed higher average DNA methylation levels when compared to the wild-type strain (Figure 5B). To confirm that this was not an artifact due to differences in strain backgrounds, we compared the level of methylation in the Δhh1 strain to a wild-type *mat a* strain, and analyzed DNA methylation levels in a second Δhh1 loss of function strain in which the *hh1* gene was inactivated by repeat-induced point mutation (Folco et al. 2003). In both cases, the level of DNA methylation was higher in the Δhh1 mutant strains (Figure 5, C and D). A search for differentially methylated regions between wild-type and ΔH1 identified only a single methylated region that was specific to the ΔH1 strain (Linkage Group VI; 309,590–319,133). This hypermethylated sequence corresponds to *Sty1-1*, a DNA transposon found in the genomes of some *N. crassa* isolates (Wang et al. 2015). It is possible that the observed increase in DNA methylation at the *Sty1-1* locus is due to a site-specific increase in DNA methylation. However, it is also possible that this is actually due to a change in copy number or location of the transposon in the ΔH1 strain background. Additional work is needed to test these possibilities.

Higher methylation levels can result when individual cytosines are methylated at a higher frequency in a population of nucleci, or when new cytosines become methylated (or a combination of both). To determine which of these possibilities was the case in H1-deficient strains, we determined the number of methylated cytosines in wild-type and Δhh1

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**Figure 3** H1-3XFLAG is depleted from gene bodies and modestly enriched in promoters of expressed genes. (A) Metaplots depict the average ChIP-seq enrichment pattern across all *N. crassa* genes for two replicate FLAG ChIP-seq experiments performed with the *hh1-3xflag-Hph* strain and a WT negative control strain (no FLAG-tagged protein). Metaplots are centered on the transcriptional start (TSS; left) or the transcriptional termination site (TTS; right). (B) All *N. crassa* genes were ranked by expression level and split into quintile groups. Quintile 1 (Q1) corresponds to genes with the highest expression level, whereas Q5 corresponds to genes with the lowest expression. The metaplot depicts the H1-3XFLAG enrichment pattern for each expression group across the TSS or TTS. (C) Heatmaps show the distribution of H1-3XFLAG across all *N. crassa* genes centered on the TSS (left) or TTS (right). Genes are ordered by expression level from highest to lowest. (D) Heatmaps show the distribution of RNA polymerase II across all *N. crassa* genes centered on the TSS (left) or TTS (right). Genes are ordered as in C. Data in B–D are from replicate one. Chip-seq; Chomatin-immunoprecipitation combined with sequencing; WT, wild-type.
using a binomial test in combination with multiple testing correction (see Materials and Methods). The number of methylated cytosines increased by \( \frac{1}{25} \) in the \( \Delta H1 \) strain. We next compared methylation frequency at cytosines that were scored as methylated in both strains. This revealed that shared sites are methylated at higher frequency in nuclei from the \( \Delta H1 \) strain. Thus, loss of \( H1 \) leads to a subtle, but global increase in DNA methylation at relics of repeat-induced point mutation.

DISCUSSION

We applied genomic and molecular methods to investigate \( H1 \) in the model fungus \( N. crassa \). We first confirmed that \( Neurospora \) \( H1 \) is a chromatin component by constructing a functional epitope-tagged \( H1 \) fusion protein and analyzing its localization in cell fractionation and ChIP-seq experiments. This revealed that \( H1 \) is globally enriched throughout the genome, occupying both heterochromatic and euchromatic regions. Surprisingly, we observed the highest enrichment of \( H1-3XFLAG \) in promoters of expressed genes. Although the overall amplitude of enrichment was low, preferential enrichment of \( H1-3XFLAG \) was clearly correlated with expression level. These results may indicate that \( H1 \) plays different roles in fungi and animals. In mouse ESCs, \( H1c \) and \( H1d \) are depleted from strong promoters and enhancers (Cao et al. 2013). It should be noted that \( H1 \) protein levels are significantly reduced in ESCs compared to differentiated cells (Fan et al. 2003), which might explain why \( H1c \) and \( H1d \) are not detected at ESC promoters. This seems unlikely, however, as ChIP-seq analysis of \( H1b \) from ESCs and from differentiated cells revealed that this protein was similarly enriched at repressed genes and depleted from active promoters (Li et al. 2012a). On the other hand, given that mammals encode multiple \( H1 \) variants, it is possible that certain \( H1 \) variants will

**Table 2** Paired-end read insert size

| Sample                | Digest Time | Mean Insert Size ± SD |
|-----------------------|-------------|-----------------------|
| Wild-type replicate 1 | 20 min      | 139.9 ± 23.7          |
| Wild-type replicate 2 | 20 min      | 144.7 ± 25.1          |
| \( \Delta H1 \) replicate 1 | 20 min | 144.1 ± 22.4          |
| \( \Delta H1 \) replicate 2 | 20 min | 139.6 ± 22.7          |
| Wild-type replicate 1 | 60 min      | 127.8 ± 33.0          |
| Wild-type replicate 2 | 60 min      | 126.5 ± 28.3          |
| \( \Delta H1 \) replicate 1 | 60 min | 129.8 ± 33.9          |
| \( \Delta H1 \) replicate 2 | 60 min | 125.6 ± 24.3          |

\[ a \pm SD. \]
bind preferentially to nucleosome-depleted promoter regions, similar to the case for *Neurospora* H1. Indeed, analysis of H1 variants by DamID showed that H1.1 exhibits a distinct localization compared to other histones and is not excluded from promoters like the other so-

Figure 5 Increased DNA methylation is observed at most N. crassa heterochromatin domains in H1-deficient strains. (A) The DNA methylation level (weighted DNA methylation level (%) (see Materials and Methods) is shown for 10 kb windows across N. crassa Linkage Group VII for wild-type, the ΔH1 mutant, and the Δdim-2 strain, which lacks all DNA methylation. (B–D) The metaplots show the average DNA methylation level across all previously identified wild-type methylated domains: (B) the wild-type mat A strain and the ΔhH1 strain; (C) the wild-type mat A strain and the ΔhH1 strain; and (D) the wild-type mat A strain and the hH1RIP2 strain. Data for at least two independent biological replicates of each strain are shown. (E) More cytosines are methylated in ΔH1. The plot shows the total number of methylated cytosines (see Materials and Methods) identified in each wild-type and ΔhH1 replicate. (F) The level of methylation at individual cytosines is higher in ΔH1. The percentage of total shared methylated sites (y-axis) vs. the level of methylation at individual cytosines (x-axis) is shown.

that the patterns observed here reflect the in vivo occupancy of H1. It will be interesting to determine if promoter regions interact with a subpopulation of H1 in which specific residues are post-translationally modified. It will also be interesting to determine if depletion of H1 from coding sequences of expressed genes depends on post-translational modification. This seems likely given that H1 proteins in plants and animals are extensively modified, much like the core histones (Harshman et al. 2013; Bednar et al. 2016; Annalisa and Robert 2015; Kotlińska et al. 2016). Moreover, it was shown that phosphorylation of H1 was linked to transcription by RNA polymerase I and II in humans (Zheng et al. 2010). An important goal for future studies will be to determine if *N. crassa* H1 is post-translationally modified and to determine if different forms of H1 exhibit distinct localization and/or distinct functions.

We found here that deletion of *hH1* from *N. crassa* did not substantially alter global MNase accessibility or nucleosome positioning. Moreover, H1 did not impact the size of protected DNA fragments produced by MNase treatment or the distance between adjacent nucleosomes in genes. These results point to clear differences in how H1 interacts with chromatin in *N. crassa* and in animals. Indeed, H1 depletion caused increased MNase accessibility, altered nucleosome spacing lengths, and reduced chromatin compaction in H1 triple-knockout ESCs (Fan et al. 2005). Our results could indicate that *N. crassa* H1 does not bind to the linker DNA and the dyad axis of the NCP as demonstrated for animal H1
Another possibility is that \textit{N. crassa} H1 is more dynamic than H1 in higher eukaryotes. FRAP studies revealed that mammalian H1 variants exist in high-mobility and low-mobility pools, and that the half-life of fluorescence recovery after H1-GFP bleaching was significantly shorter than for the core histones (Misteli et al. 2000; Lever et al. 2000). Interactions between H1 and the NCP may be even more transient in \textit{N. crassa}, such that H1 does not interfere with M\textit{N}ase digestion even though it interacts with the same region of the nucleosome protected by animal H1.

We found increased DNA methylation in H1-deficient cells of \textit{N. crassa}. H1 affects DNA methylation in both \textit{A. thaliana} and animal cells, but the relationship between H1 and DNA methylation is different in these systems. In animals, H1 variants promote repressive modifications, including DNA methylation in mammals (Yang et al. 2013) and H3K9me2 in both mammals and \textit{Drosophila} (Li et al. 2012a; Lu et al. 2013). The observation that H1-deficient cells exhibit hypermethylation demonstrates that \textit{N. crassa} H1 is not required to promote DNA methylation or H3K9 methylation, which directs DNA methylation in \textit{Neurospora} (Tamaru and Selker 2001). Similar hypermethylation was reported in \textit{A. immersus} (Barra et al. 2000). Moreover, the DNA methylation phenotypes of \textit{N. crassa} and \textit{A. immersus} are reminiscent of \textit{A. thaliana} H1 depletion lines, where a global increase in DNA methylation was observed in heterochromatin domains along with loss of DNA methylation in euchromatic transposon sequences (Wierzbicki and Jerzmanowski 2005; Zemach et al. 2013). These results indicate that H1 can limit DNA methylation in plants and fungi. Indeed, depletion of \textit{A. thaliana} H1 rescued the reduced DNA methylation phenotype of \textit{ddm1} plants, leading to the conclusion that DDM1 promotes DNA methylation by removal of H1. The \textit{Neurospora LSH/DDM1} homolog MUS-30 does not impact DNA methylation levels, therefore we predict that an \(\Delta hH1 \mu m u s-30\) double mutant would resemble the \(\Delta hH1\) single mutant strain (Basenko et al. 2016). Taken together, the data from fungi, plants, and animals may indicate that H1 evolved a new function to promote heterochromatin modifications in the animal lineage.

It is possible that \textit{N. crassa} H1 limits access of the DNA methyltransferase \textit{DIM-2} or the H3K9 MTase \textit{DIM-5\textsuperscript{SKAT}}. A recent study showed that binding of H1 to the NCP limited the dynamics and modifiability of the H3 tail \textit{in vitro} (Stützer et al. 2016), consistent with this possibility. In addition, increased accessibility of the DIM-2 DNA methyltransferase was linked to hypermethylation in the \textit{N. crassa} Histone Deacetylase-1-deficient strain (Honda et al. 2012). On the other hand, in \textit{A. thaliana}, H1 was required for imprinting of the MED\textit{EA} locus, which involves active removal of methyl cytosine bases by DNA glycosylases (Rea et al. 2012). A mechanism for DNA demethylation has not been described in \textit{N. crassa}, but it is possible that H1 promotes removal of methylated cytosines in heterochromatin domains. Additional studies are needed to understand exactly how H1 impacts DNA methylation levels in \textit{N. crassa}. Overall, this work adds to the diverse set of phenotypes that have been reported following depletion of H1 in plants, animals, and fungi. Future work to investigate H1 in fungal systems is likely to yield new insights into the evolution and the functions of this important group of proteins.

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