THE ROLE OF POST-TRANSLATIONAL MODIFICATIONS IN REGULATING DISTINCT FUNCTIONS OF HETEROCHROMATIN PROTEIN ONE

by

COLLIN HICKMANN

A THESIS

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Eric U. Selker, Ph.D.

Heterochromatin is a minimally transcribed, densely bundled complex of DNA and associated factors comprising large regions of the eukaryotic genome. It is essential for chromosome stability, genome integrity, gene regulation, and the silencing of transposons. The filamentous fungus *Neurospora crassa* is often employed as a model organism to study the epigenetic regulation of heterochromatin. In Neurospora, the conserved scaffolding protein heterochromatin protein 1 (HP1) binds H3 histones marked by lysine nine trimethylation (H3K9me3) and recruits other proteins to form at least four distinct complexes. HP1 recruits the DIM-2 DNA methyltransferase, which catalyzes DNA methylation, and the Mi-2 chromatin remodeler, which promotes centromeric silencing. HP1 is also an essential component of the HCHC complex, which facilitates histone deacetylation, and the DMM complex, which limits aberrant heterochromatin spreading. However, it is unclear how these disparate functions are coordinated. We hypothesized that they are modulated by post-translational modifications (PTMs) of HP1. Previously, we used mass spectrometry to identify HP1 sites harboring methylation, acetylation, formylation, and phosphorylation. I used amino acid substitutions at a subset of these sites to prevent individual PTMs in vivo. Substitutions at multiple sites were found to cause a substantial decrease in centromeric silencing independent of DNA methylation. These results suggest that the recruitment of Mi-2 to incipient heterochromatin may be selectively mediated by specific PTMs.

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Table of Contents

Introduction 1
- Epigenetics: The Language of Life 1
- Epigenetics and Chromatin Structure 2
- Heterochromatin Establishment in Neurospora 3
- Background Work and Hypothesis 7
- Objectives and Experimental Strategy 8
  - Objective 1: Substitute PTM Sites 9
  - Objective 2: Test the Role of HP1 PTMs in Facilitating DNA Methylation 11
  - Objective 3: Assess Heterochromatin Spreading in Mutants 11
  - Objective 4: Test Centromeric Silencing 11
- Introduction to Research Techniques 12
  - Site-Directed Mutagenesis 12
  - Molecular Cloning and Recloning 13
  - His-3-Targeting Transformation by Electroporation 14
  - Gel Electrophoresis and Purification 16
  - Blot Analysis 17

Methods 20
- Site-Directed Mutagenesis 20
- Transformation into DH5α *E. coli* 21
- Recloning 22
- Transformation into Neurospora 23
- Strains, Growth Conditions, and Crosses 24
- Southern Analysis of Genotype and DNA Methylation 24
- Western Blotting to Detect Ectopic HP1 Expression 25
- Assessment of Centromeric Silencing by Spot Testing for Basta Resistance 26

Results 28

Partial Loss of HP1-Mediated Centromeric Silencing in Strains with Substitutions at Certain PTM-Sites 29
Evidence Against a Universal Defect in HP1 Functions 31
Decreased Silencing in PTM-Site Mutants is Not Accompanied by Consistent Hypermethylation of Centromeres 35
Normal Containment of DNA Methylation at Boundaries of Heterochromatin Domains in PTM-Site Mutants 38
Discussion 40
Specific Requirement of Certain HP1 PTMs for Centromeric Silencing 40
Putative Dependency of Mi-2 Recruitment on PTMs in the Chromoshadow Domain 40
Defects in HCHC Activity 42
Tiered Phenotype Effects in Strains with Partial Loss of HCHC 42
Concomitant Antagonistic Defects in HCHC and DMM 43
Other Possible Causes of Mutant Phenotypes 44
Future Directions 46
Conclusion 48
Supplementary Information 50
Glossary 56
Bibliography 62

List of Figures

Figure 1: Chromatin Composition and Conformation 3

Figure 2: Heterochromatin Establishment in Neurospora 5

Figure 3: HP1 PTMs Detected by Mass Spectrometry 8

Figure 4: Substitution of Lysine to Prevent or Mimic Acetylation 10

Figure 5: *His-3*-Targeting Transformation by Electroporation 16

Figure 6: Schematic of Experimental Design 27

Figure 7: Reduction of Centromeric Silencing in Strains with Substitutions at PTM Sites 31

Figure 8: Decreased Silencing in PTM-Site Mutants is Not Associated with Consistent Changes in DNA Methylation at Known Heterochromatin Domains 34

Figure 9: DNA Methylation in the Pericentric Heterochromatin of PTM-Site Mutants 37

Figure 10: Control of Heterochromatin Spreading is Not Disrupted in Strains With Substitutions at Particular PTM-Sites of HP1 39

List of Tables

Table 1. Site-directed mutagenesis 21

Table 2. Generation and Testing of Strains with Substitutions in Ectopic *hpo* 28

Supplementary Table S1. Primers used in this study 50

Supplementary Table S2. *Neurospora crassa* strains used in this study 53

Supplementary Table S3. Recipes used in this study 54

Introduction

Epigenetics: The Language of Life

DNA is commonly referred to as the blueprint of life, but discoveries in the past few decades have challenged this notion. At the completion of the human genome project, many researchers were shocked to discover that the entire human genome contains only 3.2 billion base pairs, or about 770 megabytes of information[1–3]. That means a DVD has more than six times the storage capacity of your genome. Furthermore, this sequence contains only 20,000-25,000 genes. In light of this, it became clear that the information contained in the DNA sequence is only part of the picture.

We now understand that the genome functions more like a dictionary than a complete set of instructions. The dictionary is a complete catalogue of the words we use to convey ideas, but the true complexity and versatility of language is derived from our ability to choose the words we need and to connect them in a meaningful way. In an analogous manner, every cell in the body has the same DNA sequence, but they all read different parts of it. Rather than blindly following instructions in the genome, each cell precisely expresses combinations of genes that function together within the context of its environment. Much of the variation between cells in an organism and between organisms in a population is caused not by differences in DNA sequence, but by differing patterns of gene expression. However, we are just beginning to understand how cells decide which parts of the genome to read.

Epigenetics and Chromatin Structure

Epigenetics is the study of semi-heritable changes in gene expression or phenotype not directly caused by the DNA sequence. Rather, epigenetic traits are usually mediated by factors affecting the physical organization of DNA within a cell. In the nucleus of eukaryotes, DNA is stored in a complex with proteins and RNA. This complex is known as chromatin. If chromatin were unraveled, it would reveal sections of free DNA suspended between periodically spaced bundles known as nucleosomes, which are the basic unit of chromatin (Fig. 1A). At each nucleosome, the DNA strand is wound around a core of eight histones proteins, of which there are four types with two copies each: H2A, H2B, H3, and H4 (Fig. 1B). Each histone has a flexible chain of amino acids that protrudes from a more compactly folded globular core. These protrusions are known as histone tails, and they play an important role in determining chromatin structure and gene expression.

Chromatin can occur in two major conformations. Transcriptionally active genes are found in euchromatin, where the nucleosomes are loosely organized in such a way that DNA is accessible to the proteins involved in gene expression. In contrast, the nucleosomes of heterochromatin are packed together more tightly, causing nearby genes to have minimal transcription. Heterochromatin stabilizes chromosomes by forming large organizational structures including centromeres and telomeres[4,5]. In addition, the proper formation and maintenance of heterochromatin is critical to a wide variety of functions, including cellular differentiation, the regulation of gene expression, and the silencing of transposons [6–8].

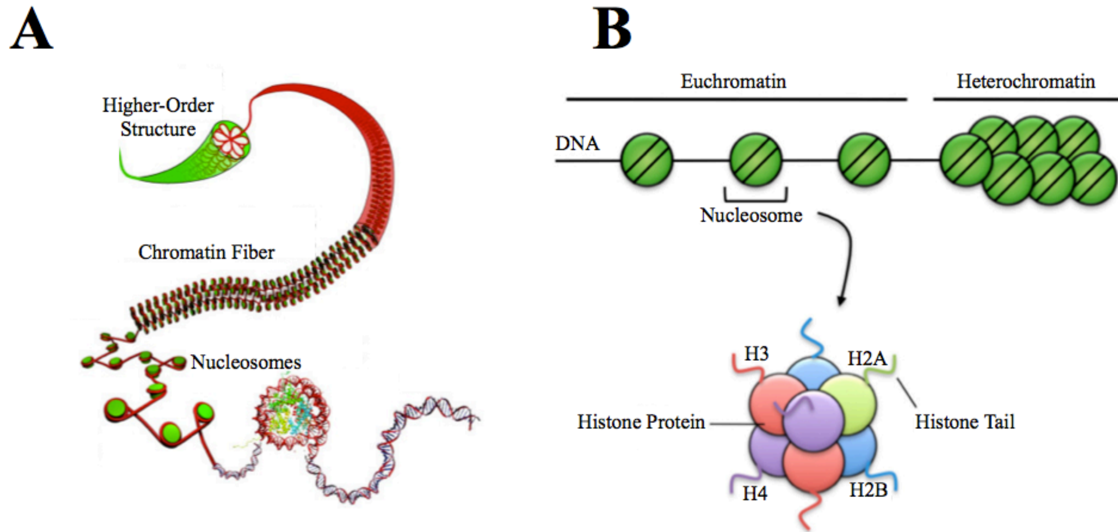

Figure 1: Chromatin Composition and Conformation

A) Chromatin is an efficient and well-organized system for storing DNA and regulating its accessibility. **B)** The two distinct states of chromatin conformation are distinguished by the spacing of nucleosomes. Figures were adopted from **A)** Iyer *et al.* 2011[9] and **B)** Russ *et al.* 2012[10].

Abnormalities affecting chromatin structure have been implicated in tumor development, autism, cardiovascular disease, and a number of other human health problems[11–13]. Despite the fundamental importance of heterochromatin for a wide variety of essential biological functions, many of the details surrounding heterochromatin formation and maintenance are poorly understood. A better understanding of the regulation of heterochromatin could provide targets for new drugs and therapeutic strategies to treat the underlying epigenetic causes of some human health problems[14].

Heterochromatin Establishment in Neurospora

The filamentous fungus *Neurospora crassa* is an ideal model organism for studying heterochromatin formation and maintenance. It shares many epigenetic

characteristics of heterochromatin with more complex eukaryotes, including humans. In Neurospora and in higher eukaryotes, cytosine methylation in DNA (5-methylcytosine, or 5mC) is one of the primary signals associated with heterochromatin[15]. Although DNA methylation is found in Neurospora, it is not required for the organism's survival. This feature allows the study of Neurospora mutants that are defective in methylation (DIM). Studies of these mutants have successfully identified a number of genes required for DNA methylation and the subsequent establishment of heterochromatin in Neurospora.

In addition to DNA methylation, several post-translational modifications (PTMs) of histone proteins are involved in the control of chromatin structure and gene expression[16]. Two of the most prominent epigenetic marks are found on lysine nine in the tail of histone H3 (H3K9). H3K9 acetylation (H3K9ac), or the addition of an acetyl group to H3K9, is associated with euchromatin and active genes[7]. This site can also be modified by the addition of a methyl group through a process fittingly known as methylation. Trimethylated H3K9 (H3K9me3), which carries three methyl groups, is a hallmark of heterochromatin and gene inactivation[17].

Almost all DNA methylation in Neurospora is found at relics of transposons that have been modified by repeat-induced point mutation (RIP). Transposons are segments of DNA that use the cell's machinery to move around the genome, sometimes creating many copies of themselves that are inserted at multiple sites. To prevent transposons from interrupting important genes, it is necessary to silence them before they have a chance to spread. For this purpose, Neurospora uses RIP, which mutates the duplicate sequences often found in transposons and produces A:T-rich regions of DNA as a

result[18]. These A:T-rich regions serve as targets for the DIM-7 protein, which is required for localization of the DIM-5 histone H3K9 methyltransferase and three other associated proteins that are collectively known as DCDC (the DIM-5/-7/-9, CUL4/DDB1 complex, Fig. 2A). DCDC mediates the DIM-5-catalyzed trimethylation of H3K9[19,20].

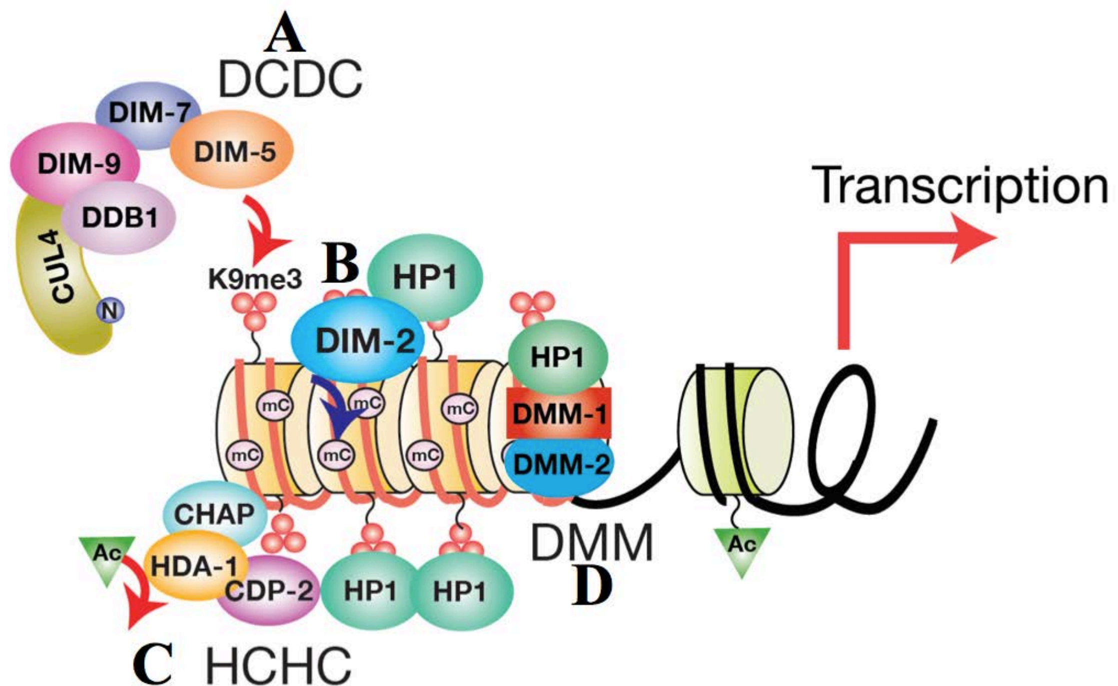

Figure 2: Heterochromatin Establishment in Neurospora

A) A:T-rich DNA is targeted by the DCDC complex, which methylates H3K9. HP1 binds H3K9me3 and recruits the following: **B)** DIM-2, a DNA methyltransferase responsible for all DNA methylation in Neurospora, **C)** the HCHC centromeric silencing complex involved in histone deacetylation, or **D)** the DMM complex, which prevents excessive heterochromatin spreading. The HP1-Mi-2 complex is not shown.

H3K9me3 is targeted by heterochromatin protein one (HP1), which is an evolutionarily conserved scaffolding protein involved in at least three distinct complexes affecting chromatin structure[21]. HP1 interacts with the DIM-2 DNA methyltransferase to mediate the methylation of nearby cytosine bases in DNA (Fig. 2A)[22]. HP1 also binds the

proteins CDP-2, HDA-1, and CHAP to form the HCHC complex, which facilitates heterochromatin establishment at centromeres and transposon relics through the deacetylation of histones at H3K9 and other sites (Fig. 2C)[21]. These functions work together to establish heterochromatin formation.

Alternatively, HP1 can form the DNA methylation modulator complex (DMM complex) by recruiting the proteins DMM-1 and DMM-2 (Fig. 2D). The DMM complex prevents H3K9me3 and DNA methylation from spreading beyond the boundaries of methylated regions and silencing neighboring genes[23], which can be required for important functions. Because it has close sequence similarity (homology) to proteins that remove methylation (demethylase proteins), it has been suggested that the DMM-1 protein may act as a histone demethylase that targets H3K9me3 at the borders of heterochromatin domains. However, *in vitro* experiments have cast doubt on this hypothesis. Regardless of the exact mechanism, the HCHC complex and DIM-2 work in parallel to promote heterochromatin formation, while the DMM complex antagonizes these functions to protect heterochromatin boundaries. More recently, the Neurospora homologue of the Mi-2 chromatin remodeler has been identified as a fourth interacting partner of HP1 (Honda *et al,* manuscript in preparation). In Neurospora and in humans, Mi-2 plays a major role in the establishment and maintenance of pericentric heterochromatin, suggesting a significant degree of functional conservation[24].

Thus, HP1 can facilitate at least four distinct functions that are essential to heterochromatin establishment and maintenance. However, the regulation of these activities is poorly understood. How does an HP1 protein recruit the correct groups to its particular position in the genome? The control of HP1's interactions with other

proteins could be the key to understanding how the activities of its associated complexes are coordinated. Ultimately, this understanding could connect the known heterochromatin complexes within a larger regulatory network that is responsive to environmental signals.

Background Work and Hypothesis

Studies in mammals, fission yeast, and Drosophila have revealed extensive modification of the HP1 protein itself, similar to that seen in histones[25,26]. Using a technique known as mass spectrometry to identify PTMs of Neurospora HP1, my mentor, Tereza Ormsby, detected widespread phosphorylation, acetylation, methylation, and formylation (Fig. 3). PTMs were detected in every functional region of the protein, but not with equal distribution. Serine phosphorylation was detected in a small cluster of two to four sites in close proximity to the terminus in both the N and C terminal domains, neither of which was detected to have any other PTM. The highest occurrence of PTMs was found in the protein's hinge region, which is a poorly conserved linker domain involved in localization to chromatin[27,28]. A number of modifications were also detected in the chromodomain (CD), which is partially responsible for binding H3K9me3[27,29], and the chromoshadow domain (CSD), which facilitates homodimerization and interactions with other proteins[30].

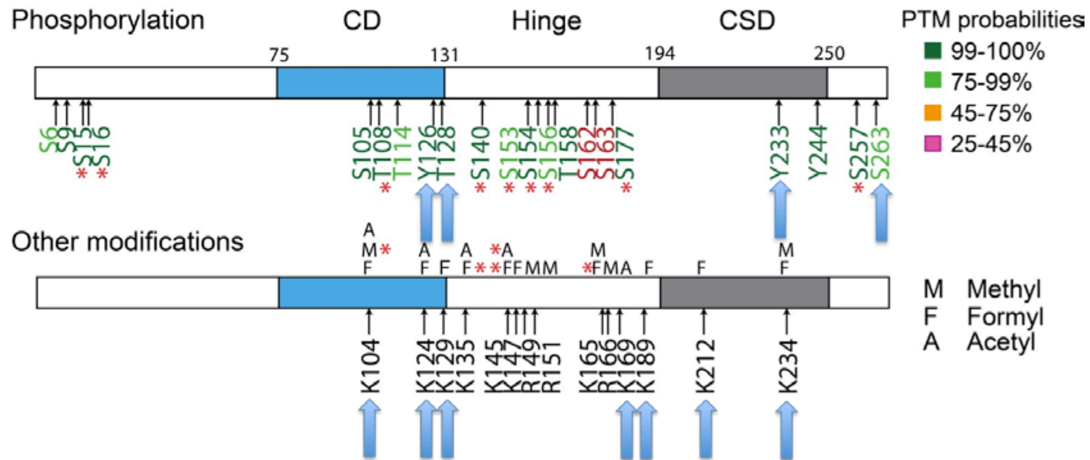

Figure 3: HP1 PTMs Detected by Mass Spectrometry

The horizontal bar represents the amino acid sequence of the HP1 protein from the N terminus on the left to the C terminus on the right. PTMs marked by a red asterisk were detected in two different facilities at which testing was performed. CD refers to the chromodomain and CSD designates the chromoshadow domain. The blue arrows indicate the subset of sites that I analyzed.

PTMs are often used to regulate protein function, and HP1 is heavily modified[31]. Furthermore, HP1's centrality in the constitutive heterochromatin pathway could allow it to behave as a fulcrum for the regulation of chromatin state; it is uniquely situated to control activity in the four downstream complexes it interacts with. This led us to hypothesize that HP1's interactions with DIM-2, the HCHC complex, the DMM complex, and Mi-2 are regulated by its PTMs. It was further hypothesized that there are multiple pools of HP1 with unique PTMs and distinct functions.

Objectives and Experimental Strategy

In this study, we aimed to determine whether PTMs are required for normal HP1 function and proper regulation of heterochromatin. Furthermore, we examined whether PTMs could influence the recruitment of HP1's interacting partners in a selective

manner, as opposed to having a more general role in HP1 function. Many proteins undergo modification, but not all are regulated by it. Some proteins undergo extensive processing before reaching their mature, functional form[32]. Are the HP1 PTMs innate to the mature HP1 protein and required for its function and stability, or do they act as a set of inducible switches that could allow HP1 to dynamically adjust its activity to local conditions or signals from a greater regulatory network? Our study design enabled us to differentiate between these two distinct schemes. To this end, our general aim was subdivided into four primary research objectives.

Objective 1: Substitute PTM Sites

To determine each PTM's role in HP1 function, it would be necessary to eliminate them individually and measure any resulting changes. However, there is no known method that can eliminate modification at a specific site *in vivo*. To work around this problem, I replaced sites suspected to harbor PTMs with other amino acids that were biochemically similar, but not subject to modification. This strategy has been successfully employed in a number of previous studies elucidating the significance of PTMs in other proteins, including in histones[33–36]. I used this strategy to prevent phosphorylation, methylation, acetylation, and formylation of a number of amino acids, and also to mimic the hyperacetylation of lysine.

Lysine is positively charged, but acetylation makes the site effectively neutral under biological conditions. Arginine is positively charged and has a very similar structure to lysine, but it cannot be acetylated (Fig. 4A). Arginine substitution was used to prevent acetylation at sites where lysine acetylation was detected. In contrast, glutamine is a polar, neutral amino acid that is structurally similar to acetyl-lysine.

Glutamine has similar properties to acetyl-lysine, but it can't be de-acetylated because it doesn't actually have an acetyl group (Fig. 4B). For this reason, glutamine substitution was chosen to mimic the constitutive acetylation of lysine.

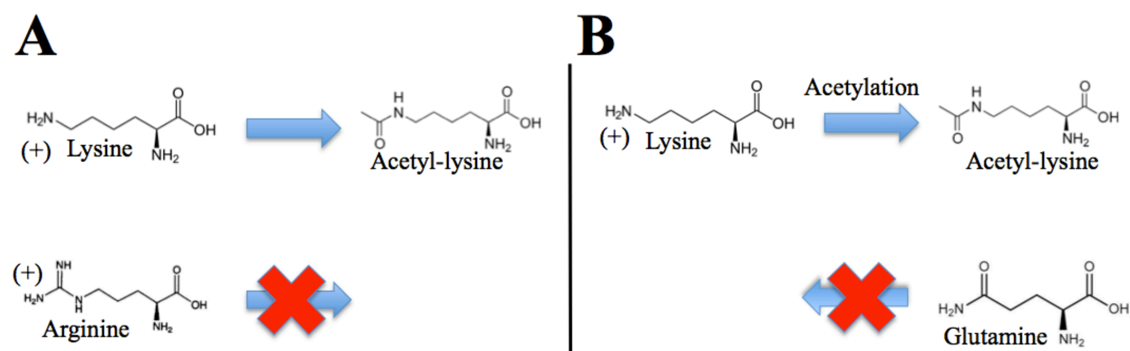

Figure 4: Substitution of Lysine to Prevent or Mimic Acetylation

A) Lysine's positive charge is reduced by acetylation. Arginine prevents acetylation and mimics lysine in its positively charged, unmodified state. **B)** Glutamine mimics the charge and structure of acetyl lysine, but it cannot be deacetylated. Glutamine substitution therefore mimics the constitutive acetylation of lysine.

Although arginine is a good mimic of unmodified lysine, it cannot be used to prevent methylation or formylation. Instead, leucine substitution was used to prevent lysine methylation and formylation at suspected PTM sites. While leucine is less biochemically similar to lysine than arginine is, it will still disrupt any interactions that depend on methylation or formylation, and based on past studies, it is unlikely to cause protein misfolding with a single substitution[33]. A similar strategy was employed to prevent phosphorylation. Phenylalanine was used to prevent the phosphorylation of tyrosine, and alanine was used to prevent phosphorylation of serine and threonine. Due to the immense scale of the project and the considerable amount of labor required for each mutant, my research focused on a subset of the PTM sites, while my mentor dealt with the remainder (Fig. 3 and Table 1).

Objective 2: Test the Role of HP1 PTMs in Facilitating DNA Methylation

Testing our research hypothesis required an evaluation of function for each of the four complexes normally containing HP1. For each mutant generated by the first objective, I assessed DNA methylation at multiple heterochromatin domains and compared it to wild-type (WT) levels. DNA methylation in heterochromatin domains was used as an indicator of DIM-2 activity. DNA methylation is lost across the genome in the absence of functional DIM-2 or HP1, while partial defects in DIM-2 activity confer a reduction in DNA methylation. Detection of normal DNA methylation can serve as decisive evidence that a missing PTM is not required for DIM-2 function. Defects in the other HP1 partners are accompanied by characteristic changes in the pattern of DNA methylation that can be used as an indicator of their activity.

Objective 3: Assess Heterochromatin Spreading in Mutants

DNA methylation was also assessed at regions that are normally euchromatic, but lie just outside of a heterochromatin domain. The DMM complex is required to limit heterochromatin spreading, so increased methylation in these border regions is indicative of DMM defects. Loss of Mi-2 causes 5mC-spreading at some regions of the genome, but it leaves the 8:G3 flanks unaffected.

Objective 4: Test Centromeric Silencing

HCHC and Mi-2 are required for normal heterochromatin formation at the centromeres. While genes are not naturally located in the pericentric heterochromatin, the state of chromatin in the centromere can be assessed by inserting a gene into the region artificially and measuring its expression[21]. The insertion of a drug resistance

gene (*bar*) into the pericentric heterochromatin enables centromeric gene expression to be ascertained by measuring resistance to the corresponding drug (Basta). While strains with WT HP1 are unable to grow on Basta due to silencing of the centromeric *bar* gene, strains with defects in the activity of HCHC or Mi-2 exhibit decreased centromeric silencing and increased growth in the presence of Basta.

A second test was used to differentiate between these two potential causes of decreased silencing in the centromere. Discrimination between the two pathways was achieved by measuring DNA methylation at the centromere. In strains with defective recruitment of the HCHC complex, the loss of centromeric silencing is accompanied by a paradoxical increase in centromeric DNA methylation[21]. This effect is not observed in Mi-2-deficient strains, so it is a useful indicator of HCHC defects (Honda *et al,* manuscript in preparation). Together, these research objectives make it possible to eliminate individual PTMs from HP1 and detect resulting changes in the activity of each of its four associated complexes: DIM-2, the HCHC complex, the DMM complex, and Mi-2.

Introduction to Research Techniques

To facilitate reading by an interdisciplinary audience, I have included this section explaining the principles of the major techniques employed in this study.

Site-Directed Mutagenesis

Site-directed mutagenesis is used to generate desired mutations at specific sites in a DNA sequence. The basis of this technique is the polymerase chain reaction (PCR), which uses the replication enzyme DNA polymerase to generate many copies of a target

sequence. DNA polymerase cannot initiate replication without primers. A primer is a short piece of DNA that binds to a matching complementary sequence in the DNA template and serves as the beginning of a new replicate strand, which is extended by DNA polymerase. Site-directed mutagenesis uses PCR primers that don't perfectly match the complementary template sequence, but still initiate replication. The errors in the primers are incorporated into the new copies of DNA. In this way, primer sequences can be designed to introduce specific mutations at predetermined sites during PCR site-directed mutagenesis.

Molecular Cloning and Recloning

Molecular cloning is a set of techniques that enables researchers to manipulate and modify DNA sequences *in vitro*. It also allows them to efficiently prepare large quantities of genetic material for experimentation. Molecular cloning takes advantage of various genetic tools, such as plasmids and specific enzymes that are used naturally by bacterial cells. Plasmids are relatively small rings of DNA that can be copied prolifically and shared between bacterial cells. This allows bacteria to exchange useful genes, like those involved in antibiotic resistance. Researchers use plasmids as a convenient vehicle to carry engineered DNA sequences (vectors) into a host organism.

While the process is highly amenable to adaptation for a wide variety of purposes, a basic cloning procedure might be performed as follows: First, plasmids are isolated from a bacterial culture. Then, the gene or sequence of interest (the insert) is incorporated into the plasmid vector. This is accomplished with restriction enzymes (endonucleases), which cleave DNA at specific target sequences called restriction sites. During cloning, a restriction enzyme is used to cleave engineered target sites that border

the gene of interest, liberating it from the extraneous surrounding DNA. The same restriction enzyme is also used to cleave the plasmid at a desired location in its DNA sequence (a locus), leaving it free to accept the gene. An enzyme-mediated reaction known as ligation joins the free ends of the gene segment with the free ends of the plasmid, effectively integrating the gene into the recircularized plasmid.

The recombinant plasmid can then be transferred back into a bacterial host through a process known as transformation. Transformation uses heat or electric shock to make the bacterial cell membrane more permeable, which causes some fraction of cells to accept foreign genetic material. Plasmid vectors are designed to include antibiotic resistance genes so that growth on antibiotic-containing media will select for cells that accepted the plasmid. The transformed bacteria will rapidly replicate the plasmid during exponential growth, allowing large quantities of it to be easily isolated. The recombinant gene sequence can further be excised and recloned into other vectors if desired.

His-3-Targeting Transformation by Electroporation

While genes can be easily transformed into bacteria as plasmids, many eukaryotes do not use plasmids, and they are not imported into the nucleus of Neurospora[37]. Foreign DNA must be integrated into the nuclear genome if it is to become a stable part of the genotype of Neurospora. *His-3*-targeting transformation is a technique that allows genes to be inserted into the Neurospora genome just downstream of the *his-3* gene, which encodes an enzyme required for the biosynthesis of the amino acid histidine. This technique takes advantage of genetic recombination. Recombination is normally used to exchange DNA segments between homologous chromosomes, but it

can also facilitate crossover between any two DNA strands with close sequence similarity (homology).

To promote recombination into the *his-3* locus, a gene of interest is first recloned into the *his-3*-targeting plasmid pBM61. Plasmid pBM61 contains a truncated copy of the *his-3* gene and a multiple cloning site with restriction sites for insertion of the desired gene followed by a segment of 3' flanking, non-coding sequence from linkage group I (LGI), which is just downstream of the *his-3* gene.

To force Neurospora to accept recombinant sequence, we use a process known as electroporation, during which a small electrical current is applied to permeabilize asexual Neurospora spores (conidia)[38]. The recipient conidia are engineered to have a defective copy of *his-3* with a loss-of-function mutation near the 3' end of the gene. Consequently, they require histidine supplementation for growth. In a small fraction of the electroporated cells, recombination of the defective, endogenous *his-3* gene with the *his-3*-targeting DNA will generate a functional version of the *his-3* gene (Fig. 5A). A second crossover completes the insertion by linking the 3' flanking sequence of *his-3* with the rest of linkage group I (Fig. 5B). The transformed cells are grown on agar plates in the absence of histidine to select for cells in which the function of *his-3* was restored through a double crossover that must also integrate the gene of interest into the genome.

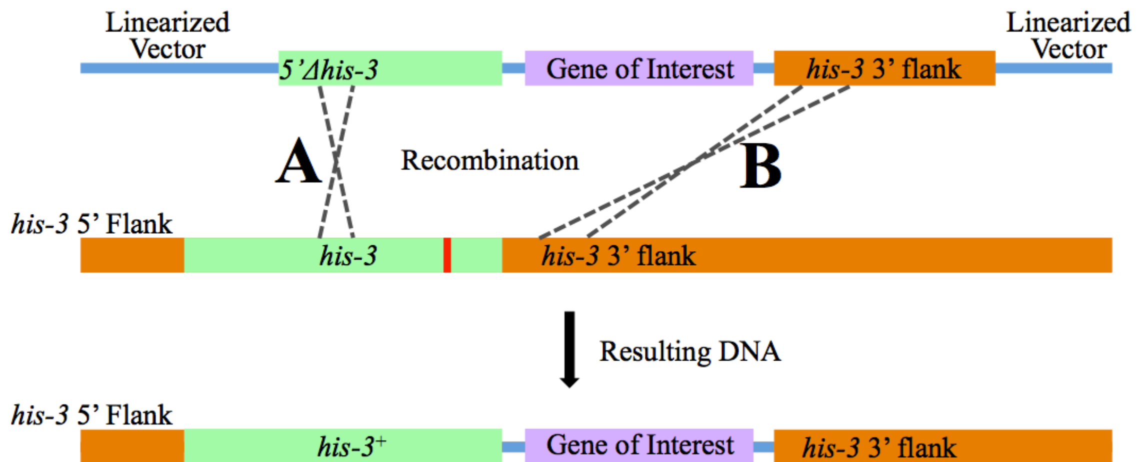

Figure 5: *His-3*-Targeting Transformation by Electroporation

A) *5'Δhis-3* designates a truncation at the 5' end of a *his-3* gene that is otherwise functional. The red stripe in the lower *his-3* indicates a loss of function mutation. Recombination between the two restores the function of *his-3*. **B)** Sitting downstream of the desired gene, a duplicate segment of the *his-3* 3' flank recombines back into its endogenous locus, carrying the gene with it. The reciprocal recombination product is not depicted in this illustration, because it is not integrated into the Neurospora genome.

Gel Electrophoresis and Purification

Gel electrophoresis is a technique used to separate biological macromolecules based on their size and charge. The technique can be used to analyze samples containing a mixture of proteins or distinct fragments of DNA or RNA. During electrophoresis, a rectangular gel slab composed of agarose or polyacrylamide is commonly submerged beneath a buffer solution, although other setups with gel columns or capillary tubes can also be used. Liquid samples are loaded into small "wells," or gaps in the gel, and an electric current is run across it horizontally. Under the conditions most commonly used for electrophoresis, the biological macromolecules are negatively charged, and will migrate towards the positive electrode (cathode). To move towards the cathode, macromolecules must squeeze through microscopic pores in the gel. Smaller molecules move more rapidly through the gel because they encounter less

resistance than large molecules. Through this process, dissimilar fragments in a sample will separate into distinct bands based on their size and charge, with each band containing many copies of a single fragment or multiple fragments with similar size and charge. While DNA is typically electrophoresed in agarose gels, proteins are most commonly separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The banding pattern of DNA or protein fragments can be visualized with a variety of stains. The distance a molecule travels through the gel is inversely related to its size, and the number of bands in the gel is equal to the number of fragments with unique size, although this is not always discernable. In addition, highly concentrated fragments will produce thick bands that stain heavily when compared to fragments occurring in smaller amounts. In this way, gel electrophoresis can often be used to count the different fragments in a sample, determine their approximate sizes, and estimate their relative concentrations. Electrophoresis is also useful for purification. After the molecules in a sample separate by size, fragments of the desired size can be excised from the gel, and the DNA or protein can be purified from them.

Blot Analysis

Although the fragments in a gel can be visualized by staining after electrophoresis, the available stains cannot selectively bind to a single desired macromolecule. In some situations, like the electrophoretic analysis of a lysate with hundreds of different proteins, or of genomic DNA digested by a restriction enzyme to give thousands of DNA fragments, all of the bands will blur together to produce a continuous smear that obscures the desired signal. Blot analysis is a technique that can

be used to visualize only the fragment of interest. I used two different types of blots during the course of this study, which are distinguished by the class of macromolecule they analyze. "Southern blots" are used for DNA samples, while "western blots" are used for proteins[39–41].

Despite some procedural differences, these blots all use the same basic principle. After segregation during electrophoresis, macromolecules are drawn out of the gel by capillary force or electric current. The fragments are deposited on a high-affinity binding membrane resting on the gel. The blotting membrane retains the same banding pattern as the electrophoresed gel. However, the membrane leaves these macromolecules accessible to direct interaction with reagents that can visualize them in a much more selective manner.

Proteins on a western blot are selectively visualized with specialized antibodies that recognize and bind to a specific protein sequence, known as an epitope[40,41]. Genetic engineering can be used to physically link proteins of interest with distinctive epitopes, which are referred to as affinity tags (e.g. FLAG tag)[42]. To facilitate detection of tagged proteins, antibodies are commonly linked to the horseradish peroxidase (HRP) enzyme. HRP catalyzes the chemiluminescence reaction between luminol and hydrogen peroxide, producing a detectable light signal wherever the antibody binds its target protein. I used western blot analysis to verify expression of the FLAG-tagged HP1 protein in engineered Neurospora strains.

Hybridization probes, which contain complementary sequence to a desired fragment of DNA, are used to visualize specific nucleotide sequences in Southern blots. Probes are synthesized by a PCR reaction that incorporates the radioactive

phosphorus-32 isotope (^{32}P) into the reaction product. The resulting probe will selectively bind to the target sequence and impart radioactivity to the corresponding bands on the membrane. Exposure of a photosensitive film or phosphor screen allows these specific bands to be visualized.

In conjunction with a restriction digest, this technique has a number of practical applications. Southern analysis can be used to determine whether an insert has been integrated between known restriction sites at a desired locus (such as *his-3*). Such an insertion will cause a predictable increase in the length and molecular of mass of the segment between the two sites, which is excised during the appropriate restriction digest. Probing a Southern blot for the planned insertion site and evaluating the band migration distance can detect this increase.

A variant of this strategy uses Southern analysis to assess DNA methylation. In this method, a DNA sample is digested separately with two different restrictions enzymes that target the same sequence (isoschizomers). One of the enzymes cleaves its target regardless of cytosine DNA methylation, but the other can only cut unmethylated restriction sites, and is said to be 5mC-sensitive. The isoschizomers *DpnII* (cytosine methylation-insensitive) and *BfuCI* (cytosine methylation-sensitive) are commonly used for this purpose. If a locus is methylated, Southern analysis will reveal larger fragments in the *BfuCI* digest that are not present in the *DpnII* digest, along with a corresponding decrease of smaller fragments in the *BfuCI* digest. The level of DNA methylation at a site is deduced from the ratio of large (methylated) and small (unmethylated) fragments in the *BfuCI* digest. By probing for a variety of regions across the genome, patterns of DNA methylation can be established and compared between different samples.

Methods

Site-Directed Mutagenesis

For site-directed mutagenesis, my mentor prepared a construct of *hpo* in the pBluescript KS+ vector (pBSK from Stratagene), which is smaller and more conducive to PCR mutagenesis than other available plasmids (Fig. 6A)[43]. The *hpo* template *phpo::hpo-10xGly-HAT-3xFLAG,* containing the HP1 gene (*hpo*) fused to a 10-glycine tail linker, a histidine affinity tag (*HAT*), and the 3-FLAG affinity tag (*3xFLAG*), all under the control of the endogenous HP1 promoter (*phpo*), was inserted between the *NotI* and *ApaI* restrictions sites of pBSK. I used this construct as a template to generate all *hpo* mutants listed in Table 1.

The following protocol was derived from the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene. For each desired mutation, 2 single-primer PCR reactions were set up in parallel using complementary primers with the appropriate mismatch (Tables 1 and S1). The reaction mix contained 25 ng of the template plasmid, 10 pmol of primer, 0.5 mM dNTPs, and 2 units of Phusion DNA Polymerase (Thermo Fischer Scientific) in a 50 µl volume of 1x Phusion HF buffer. The single primer reactions were carried out under the following conditions in a T300 thermocycler (Biometra): initial denaturation at 98 °C for 30 seconds, followed by 4 cycles of 98 °C for 10 seconds, 60 °C for 1 minute, and 72 °C for 12 minutes, then a final extension at 72 °C for an additional 7 minutes. Then, I combined 25 µl of product from each of the complementary reactions in a single tube with an additional unit of Phusion DNA polymerase. Another 18 cycles were conducted as before, but with an annealing temperature of 65 °C held for 30 seconds each cycle.

Following the PCR reaction, I added 10 units of the *DpnI* endonuclease (New England Biolabs) and incubated at 37 °C for 4 hours. *DpnI* digests the Dam-methylated parental strands, but leaves the unmethylated mutant copies intact. Mutagenized plasmids were purified with the MinElute PCR Purification Kit (Qiagen) and eluted in 15 µl of water in accordance with the manufacturer's protocol.

Table 1. Site-directed mutagenesis

Mutation	Suspected PTM	Effect of Mutation
K104R	Acetylation, Methylation, Formylation	Prevents acetylation
K104Q	Acetylation, Methylation, Formylation	Mimics hyperacetylation
K124L	Acetylation, Formylation	Prevents acetylation and formylation
K124R	Acetylation, Formylation	Prevents acetylation
K124Q	Acetylation, Formylation	Mimics hyperacetylation
Y126F	Phosphorylation	Prevents phosphorylation
T128A	Phosphorylation	Prevents phosphorylation
K129L	Formylation	Prevents formylation
K169R	Acetylation	Prevents acetylation
K169Q	Acetylation	Mimics hyperacetylation
K189L	Formylation	Prevents formylation
K212L	Formylation	Prevents formylation
Y233F	Phosphorylation	Prevents phosphorylation
K234L	Methylation, Formylation	Prevents methylation and formylation
S263A	Phosphorylation	Prevents phosphorylation

List of PTM-site substitutions generated by PCR site-directed mutagenesis and their biochemical effects. Mutagenesis primers are listed in Table S1.

Transformation into DH5α *E. coli*

For each reaction, 5 µl of purified PCR product was added to 50 µl of chemically competent DH5α *Escherichia coli* (New England Biolabs). The mixture was incubated on ice for 20 minutes, then heat shocked at 42 °C for 1 minute and allowed to recover in 1 ml SOC medium at 37 °C for 1 hour. Transformed cultures were

centrifuged at 5,000 rpm for 3 minutes, excess medium was discarded, and the resuspended concentrate was spread on agar plates with LB medium (LB agar) and ampicillin. The plates were grown overnight at 37 °C.

The next day, I picked 3-5 colonies from each plate and used them to inoculate 5 mL cultures of LB broth with ampicillin. The liquid cultures were grown overnight with constant agitation at 37 °C. Plasmids were isolated from the liquid cultures using the QIAprep Spin Miniprep Kit following the manufacturer's instructions (Qiagen). Sanger sequencing was used to confirm the presence of desired mutations. To achieve full sequencing coverage of the gene, each plasmid was sequenced with 2 separate primers: one annealing 82 base pairs upstream of the *hpo* start codon (Primer 4539, Table SI), and the other at nucleotide 450 of the coding sequence (HP1(+450)_seq_Fwd, Table S1). Sequencing was performed at the Sequetech facility (Mountain View, CA). In addition to the planned mutants, a double-mutated K189L, K212L construct was detected in one of the cultures transformed with the products of the K212L reaction. This unexpected mutant was processed and analyzed with the others.

Recloning

To facilitate recombination into the Neurospora genome at the *his-3* locus, the mutagenized *phpo::hpo-10xGly-HAT-3xFLAG* was recloned into the *his-3*-targeting pBM61 vector (Fig. 6B, pBM61 from FGSC)[38]. To this end, the inserts were excised from pBSK by *NotI-HF* and *PacI* (both from New England Biolabs). All enzyme reactions in this study were performed under the manufacturer's recommended conditions unless otherwise specified. The pBM61 vector was liberated from the WT *hpo* insert in p2905 by digestion with the same restriction enzymes. As a precaution

against self-ligation and exclusion of the insert, I used Antarctic Phosphatase (New England Biolabs) to dephosphorylate the 5' ends of the vector. Inserts and vector were isolated from the restriction products by electrophoretic gel purification with the MinElute Gel Extraction Kit (Qiagen).

Ligation was performed with 50 ng of vector, 34 ng of insert, and 400 units of T4 DNA ligase (New England Biolabs) in a 15 µl reaction volume with the appropriate buffer. After 16 hour incubation at 16 °C, the enzyme was denatured at 75 °C for 15 minutes. 5 µl of the ligase reaction was used for transformation of DH5α as described above. 3-5 colonies were picked from the LB agar ampicillin plate, then DNA was isolated from the liquid cultures and analyzed by Sanger sequencing to confirm the expected genotype as before.

Transformation into Neurospora

To facilitate recombination, constructs were linearized with *DraI*, and the smaller fragment, containing the *hpo* insert (*5'Δhis-3::phpo::hpo-10xGly-HAT-3xFLAG*), was isolated by gel electrophoresis and purified with the MinElute Gel Extraction Kit (Qiagen). An aliquot of plasmid p2905 was also processed in this manner, producing a WT *hpo* insert to be used as a control in later experiments. As previously described in the Introduction, the linearized sequences were inserted into the *his-3* locus of the N5580 strain (*mat a his-3; Δhpo::hph*) by electroporation and subsequent homologous recombination (Figures 5 and 6C)[38]. To facilitate selection of the correct progeny, transformed conidia were plated on agar containing Vogel's Medium N and sorbose (FGS Vogel's, Table S3) without supplemental histidine, then incubated at 32 °C for 4 days. From each construct, 20 transformants were randomly

selected and tested for correct integration by restriction digest and Southern analysis. I confirmed the ectopic expression of HP1 by western blotting (as described below).

Strains, Growth Conditions, and Crosses

All Neurospora strains used in this study are listed in Table S2. Recipes for media and buffers are listed in Table S3. Strains were grown, maintained, and crossed by conventional methods under standard conditions, which are detailed in past publications[44–46]. Primary transformants with proper genotype and confirmed ectopic expression of HP1 were crossed with the N4890 strain (*mat A his-3; CenVIR::bar trp-2*) to generate progeny that could be assessed for centromeric silencing by testing resistance to Basta (Fig. 6D)[21]. Crosses were performed on SC agar crossing medium using the primary transformants as the female strains. Progeny were grown on FGS Vogel's with hygromycin and tryptophan in the absence of histidine to select for the *his-3*$^{+}$-linked *hpo* construct and the knockout of endogenous *hpo* (*Δhpo::hph*).

Southern Analysis of Genotype and DNA Methylation

Progeny retaining the *CenVIR::bar* marker were identified by Southern analysis of genomic DNA samples digested with *BamHI* and visualized with a *bar* probe. Correct integration of the *hpo* construct was verified by Southern analysis of an *EcoRI* genomic digest using a *his-3* probe[38,47]. Similarly, I assessed DNA methylation by digesting DNA samples with 5mC-insensitive *DpnII*, or its 5mC-sensitive isoschizomer *BfuCI*, which leaves cytosine-methylated restriction sites intact (Fig. 6F). Southern hybridization with various probes was used to visualize the degree of fragmentation and the corresponding level of DNA methylation at numerous sites of interest[48,49]. Strains to

be analyzed were grown for 2 days under constant agitation at 32 °C in 5 mL of Vogel's medium N with 1.5% sucrose and required supplements. The methods for isolation of genomic DNA and Southern hybridization were consistent with previous research[39,44,49,50]. Probes were made by PCR of genomic DNA with the primers listed in Table S1. Restriction digests for Southern blots were performed overnight at 37 °C with 1 µg of genomic DNA and 10 units of the indicated enzyme in 15 µl of the respective buffer.

Western Blotting to Detect Ectopic HP1 Expression

Strains were grown for 16 hours under the same conditions used for genomic DNA cultures. Mycelium was collected by vacuum filtration, and excess moisture was removed by blotting with paper towels. Each sample was frozen in liquid N_2 and suspended in a volume of ice-cold lysis buffer roughly proportional to its mass (~ 9 µl/mg). Lysis was induced using a sonicator (Branson Sonifier-450) at a duty cycle of 80 with output set to 2 for three 20 second intervals interspersed by 10 minute periods on ice. Cell debris was pelleted by centrifugation at 14,000 rpm for 5 minutes at 5 °C. The supernatant was mixed with an equal volume of 2x Laemmli buffer, heated at 95 °C for 5 minutes, and reduced with 1% DTT added after heating.

Supernatants were loaded onto either 10% or 12% polyacrylamide gels and analyzed by SDS-PAGE followed by western blotting on polyvinylidene difluoride (PVDF) membranes as described previously[22]. Membranes were blocked with 3% (w/v) skim milk powder in TBS-T solution to prevent antibodies from binding the membrane in a nonspecific manner. After blocking, membranes were treated overnight with a 1:10,000 dilution of mouse monoclonal anti-FLAG M2 peroxidase (A8592 αFLAG-

HRP, Sigma) in TBS-T with 1% milk at 4 °C. Membranes were washed 3 times for 10 minutes with TBS-T solution and developed with SuperSignal West Pico chemiluminescence substrate (Thermo Fischer Scientific) according to the manufacturer's instructions. Images were captured with the Odyssey FC Imaging system (LI-COR) on the 700 nm chemiluminescence channel.

To control for protein loading, membranes were subsequently stained for phosphoglycerate kinase 1 (PGK1). First, peroxidase was inactivated by freezing the membrane and incubating with 1% NaN_3, 1% (w/v) skim milk powder in TBS-T washing buffer for 1 hour at room temperature. The blots were probed with a 1:5,000 dilution of rabbit polyclonal antibody for PGK1 (kindly provided by Stevens Lab, University of Oregon) in TBS-T with 1% (w/v) skim milk powder. Blots were washed and incubated with goat anti-rabbit-HRP diluted from a 10,000x stock in the same washing buffer, then developed and visualized as described above.

Assessment of Centromeric Silencing by Spot Testing for Basta Resistance

Expression of the *CenVIR::bar* marker was determined by spotting serial dilutions of conidia on plates with varying concentration of Basta (Bayer Cropscience, Fig. 6E). Conidial suspensions were quantified with a hemocytometer and standardized to have 10^5 conidia per 3 µl spot (3.3 x 10^4 conidia per µl). Standardized stocks were used to make three 1:10 serial dilutions. 3 µl drops from each suspension were spotted on Basta medium with varying concentrations of the drug. Plates of this medium were prepared with no drug, 1x Basta (8 mg/ml), 1.5x Basta (12 mg/ml), and 2x Basta (16 mg/ml, data not shown). Plates were imaged with a Molecular Imager Gel Doc XR imaging system (Bio-Rad) after 3 days of growth at 32 °C.

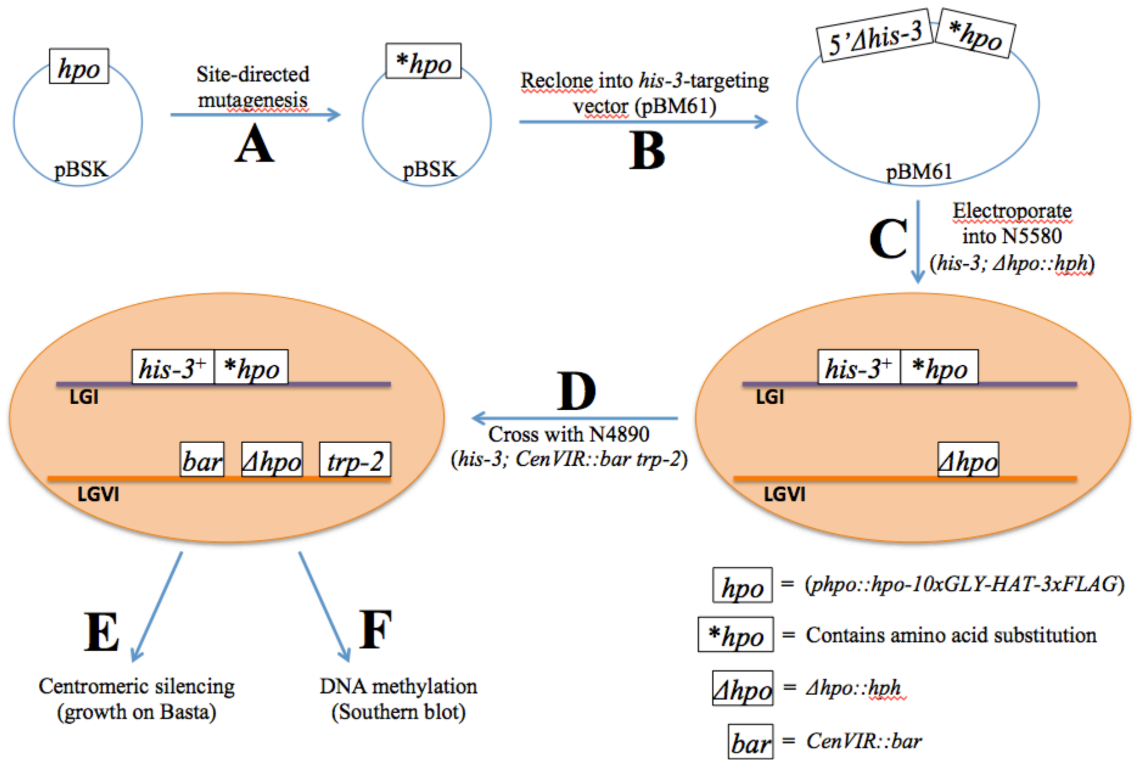

Figure 6: Schematic of Experimental Design

A) PCR site-directed mutagenesis was used to generate the desired amino acid substitutions in a small plasmid (pBSK) carrying the *hpo* construct. **B)** The *hpo* insert was excised from the pBSK vector and ligated into the pBM61 backbone of p2905 just downstream of the 5'-truncated *his-3*-gene. **C)** Constructs were linearized with and transformed into the N5580 Neurospora strain, in which the endogenous *hpo* had been replaced with the *hph* drug resistance gene. **D)** Primary transformants were crossed with the N4890 strain to produce homokaryotic progeny with the *bar* gene inserted into the pericentric heterochromatin of LGVI. **E)** Expression of the *CenVIR::bar* marker was used to assess centromeric silencing, as determined by growth on medium containing Basta-containing medium. **F)** Southern hybridizations of genomic DNA digested with a 5mC-sensitive enzyme were probed for various regions of the genome to investigate patterns of DNA methylation.

Results

Table 2. Generation and Testing of Strains with Substitutions in Ectopic *hpo*

Construct	Integrated at *his-3* in N5580	*CenVIR::bar* progeny isolated	5mc and centromeric silencing tested
hpo^{WT}	yes	1	yes
hpo^{K104R}	yes	0	no
hpo^{K104Q}	yes	0	no
hpo^{K124L}	yes	0	no
hpo^{K124R}	yes	0	no
hpo^{K124Q}	yes	0	no
hpo^{Y126F}	yes	0	no
hpo^{T128A}	yes	0	no
hpo^{K129L}	yes	0	no
hpo^{K169R}	yes	1	yes
hpo^{K169Q}	yes	1	yes
hpo^{K189L}	yes	1	yes
hpo^{K212L}	yes	2	yes
$hpo^{K189L, K212L}$	yes	1	yes
hpo^{Y233F}	yes	0	no
hpo^{K234L}	yes	0	no
hpo^{S263A}	yes	0	no

We explored the functional role of several suspected PTMs of HP1 using a site-directed mutational analysis. I generated 17 FLAG-tagged *hpo* constructs with mutations at 11 suspected PTM sites under the control of the endogenous promoter (Table 2). All 17 constructs were inserted at the *his-3* locus of an *hpo* knockout Neurospora strain (N5580) in an effort to test their rescue of the *Δhpo* phenotype and compare it to the WT HP1 phenotype. At present, transformants carrying 7 of these constructs have been crossed to give progeny with the *CenVIR::bar* marker and the desired construct. I was successful in engineering strains with ectopic expression of mutant HP1 in a *Δhpo; CenVIR::bar* background, but most of these constructs have only been verified and

assessed in a single strain. Replication of the results in a larger number of progeny is currently underway, and I am also working to complete the screening and characterization of the untested mutants.

Partial Loss of HP1-Mediated Centromeric Silencing in Strains with Substitutions at Certain PTM-Sites

To determine whether PTMs play a role in HP1-dependent centromeric silencing, I analyzed expression of the centromeric *bar* marker (*CenVIR::bar*) in each of the mutant strains. Resistance to Basta was compared to control strains with normal centromeric silencing or with known defects in silencing. Control strains with knockouts of *hpo* (N4891) or *hda-1* (N4906) displayed growth on Basta at both of the concentrations tested (Fig. 7). As an additional control, I used a strain with *his-3::hpo*$^{\mathrm{T108A}}$ (N5891). In previous experiments by my mentor, this mutation was found to cause loss of silencing and DNA methylation (T. Ormsby, unpublished). The vigorous growth of these controls shows the ability of this assay to reveal defects in silencing. In contrast, a strain containing just *CenVIR::bar* (N4890) was unable to grow in the presence of Basta. Multiple control strains carrying the WT *hpo* construct at *his-3* were also tested. *hpo*^{WT}-1 (N5889) has the same lineage as *hpo*^{T108A}, and was also generated by my mentor. I produced *hpo*^{WT}-2 (N6291) by the previously described methods. These controls had minimal growth on Basta, indicating rescue of WT silencing and negligible disruption from the affinity tags or expression at the non-native locus.

However, strains with PTM-site substitutions exhibited significant reductions in centromeric silencing relative to the *hpo*^{WT} controls. Every *hpo* mutant tested displayed

a greater resistance to Basta than any of the *hpo*^{WT} controls, which is especially evident on 1x Basta. Although the mutants had greater growth on Basta relative to the *hpo*^{WT} controls, they have less resistance than the *Δhpo*, *Δhda-1*, or *hpo*^{T108A} controls, and are much more sensitive to higher concentrations of the drug (1.5x, 2x not shown).

While some mutations appeared to have a larger effect than others, the differences between mutants were obscured by high variation between sister progeny with the same *hpo* sequence. This variation is evident in the sister *hpo*^{K212L} strains, where *hpo*^{K212L}-1 has much greater resistance to Basta than *hpo*^{K212L}-2. The cause of this variation is uncertain, as is the true characteristic phenotype caused by this mutation. Unexplained variation between sister progeny has also been observed in other *hpo* mutants generated by my mentor (T. Ormsby, unpublished). While it does cause difficulty in comparing subtle differences between mutants, high phenotypic variability is not unusual for epigenetic effects, and does not necessarily negate the decrease in centromeric silencing observed in all of the *hpo* mutants I examined[51]. Using the same methods, my mentor found no consistent changes in silencing among dozens of phosphorylation site mutants (T. Ormsby, unpublished findings). This suggests centromeric silencing is sensitive to mutations at only a subset of PTM-sites, and that the observed defects may be specifically caused by loss of methylation, formylation, and acetylation at the sites I examined, and possibly at additional sites that remain to be tested. These results are highly suggestive, but they must be replicated in a larger number of progeny before they are conclusive. Results from additional progeny will determine the average effect of each mutation and overcome the observed variability.

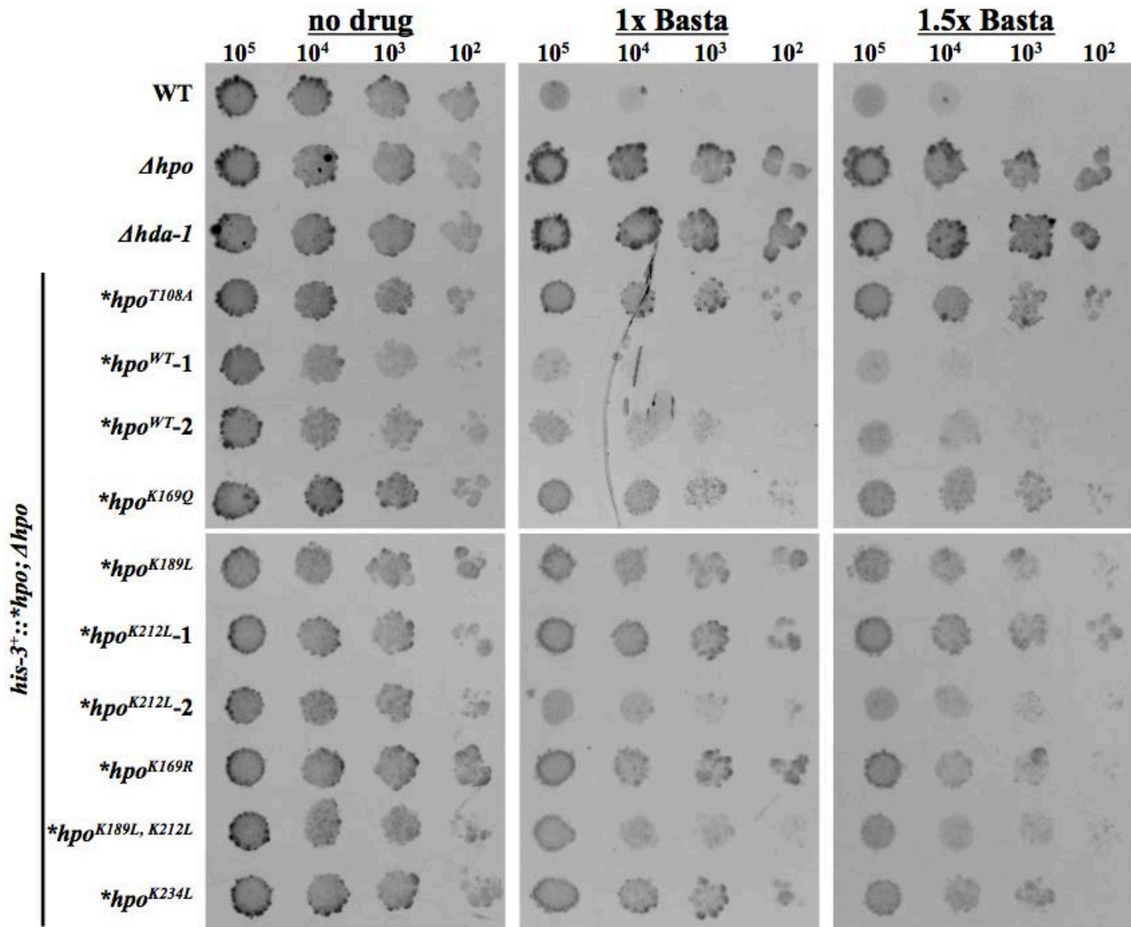

Figure 7: Reduction of Centromeric Silencing in Strains with Substitutions at PTM Sites

Standardized serial dilutions of condial suspensions from strains harboring a centromeric *bar* construct, *CenVIR::bar*, were spotted on medium containing no drug, 1x Basta (8 mg/ml), or 1.5x Basta (12 mg/ml). *hpo*^{T108A} - *hpo*^{K234L} denote substitutions in *phpo::hpo-10xGly-HAT-3xFLAG* at the *his-3* locus in a *Δhpo* background. *hpo*^{T108A} *and hpo*^{WT}*-1* were provided by my mentor, Tereza Ormsby. The curved line segment in the upper middle panel is a fracture in the agar medium resulting from a handling error. From top to bottom, the following strains were used: N5580, N4890, N4891, N5891, N5889, N6291, N6292, N6293, N6294, K212L-2, N6296, N6297, K234L.

Evidence Against a Universal Defect in HP1 Functions

Loss of centromeric silencing has previously been observed in knockouts of HCHC and Mi-2, but it may also result from a more general loss of HP1 function (as in

the case of *hpo*^{T108A}, Ormsby *et al,* unpublished). For proper centromeric silencing to occur, HP1 must be stably expressed, correctly folded, imported into the nucleus, coupled into homodimers, and properly localized to H3K9me3. Defects in any of these processes could be responsible for the observed decreases in silencing, but they would also be expected to cause other functional impairments. To discern the true mechanisms by which these substitutions disrupt centromeric silencing, we investigated their effects on cytosine methylation by Southern analysis.

First, we examined whether DNA methylation was preserved at the known heterochromatin domains 8:A6 and 8:F10 (Fig. 8). Knockout strains for *hpo* (N5580), *dmm-1* (N3064), and *hda-1* (N3610) were used as controls, along with a WT strain (N3752), and the *hpo*^{WT}-2 strain used in the silencing assay. Consistent with previous findings, *Δhpo* and *Δhda-1* caused hypomethylation at 8:A6, as indicated by the absence of larger restriction fragments relative to WT. *Δdmm-1* produced an opposite effect, causing an increased proportion of high mass *BfuCI* fragments indicative of hypermethylation from heterochromatin spreading, as seen previously[23]. An increase in the smallest fragments (0.6 kb) is also visible. This pattern is produced by a combination of central hypomethylation and peripheral hypermethylation characteristic of DMM mutants. Comparison with these controls will help to differentiate between defects in distinct functions of HP1.

The control strain expressing affinity-tagged WT HP1 at *his-3* (*hpo*^{WT}-2) has moderate hypomethylation, which suggests some degree of interference from the affinity tags. Incomplete rescue of DNA methylation from the recombinant *hpo*^{WT} could also be caused by faulty expression at the *his-3* locus, e.g. if distant enhancers of HP1

were excluded from the vector. Expression of HP1-FLAG was confirmed by western analysis of each strain (data not shown), but we cannot yet compare it to endogenous levels of expression because we do not have an antibody for the untagged protein in WT. This reduced level of DNA methylation serves as the background against which methylation in the *hpo* mutants will be measured. Relative to this control, the hpo^{K169Q} strain exhibits a minor increase in one of the larger fragments (~ 1.8 kb) and in the larger of the two smaller fragments (~ 1.2 kb). This may suggest mild hypermethylation, although it still exhibits less DNA methylation than the WT strain (N3752). A modest reduction in 8:A6 methylation was detected in the hpo^{K189L} strain. These small changes may not be significant, and would have to be reproduced in a larger number of progeny to prove that they are not the result of variance from other factors, as demonstrated by the results of the silencing assay. I found no detectable changes in DNA methylation at 8:A6 in the remaining strains.

Probing for the 8:F10 heterochromatin domain revealed no major differences between the N3752 WT, the hpo^{WT}, and the PTM-site substitution strains (Fig. 8B). There may be some hypermethylation in hpo^{K169Q}, but if so, it is too subtle to clearly discern. It is worth noting that DNA methylation at this locus is generally more robust than methylation at 8:A6. It does not require HCHC, as evidenced by the normal DNA methylation in the *hda-1* knockout. However, 8:F10 methylation does require localization of the DIM-2 DNA methyltransferase. Invariant levels of 8:F10 methylation suggest that the mutations did not disrupt the stability of HP1, its localization to H3K9me3, or its interaction with DIM-2.

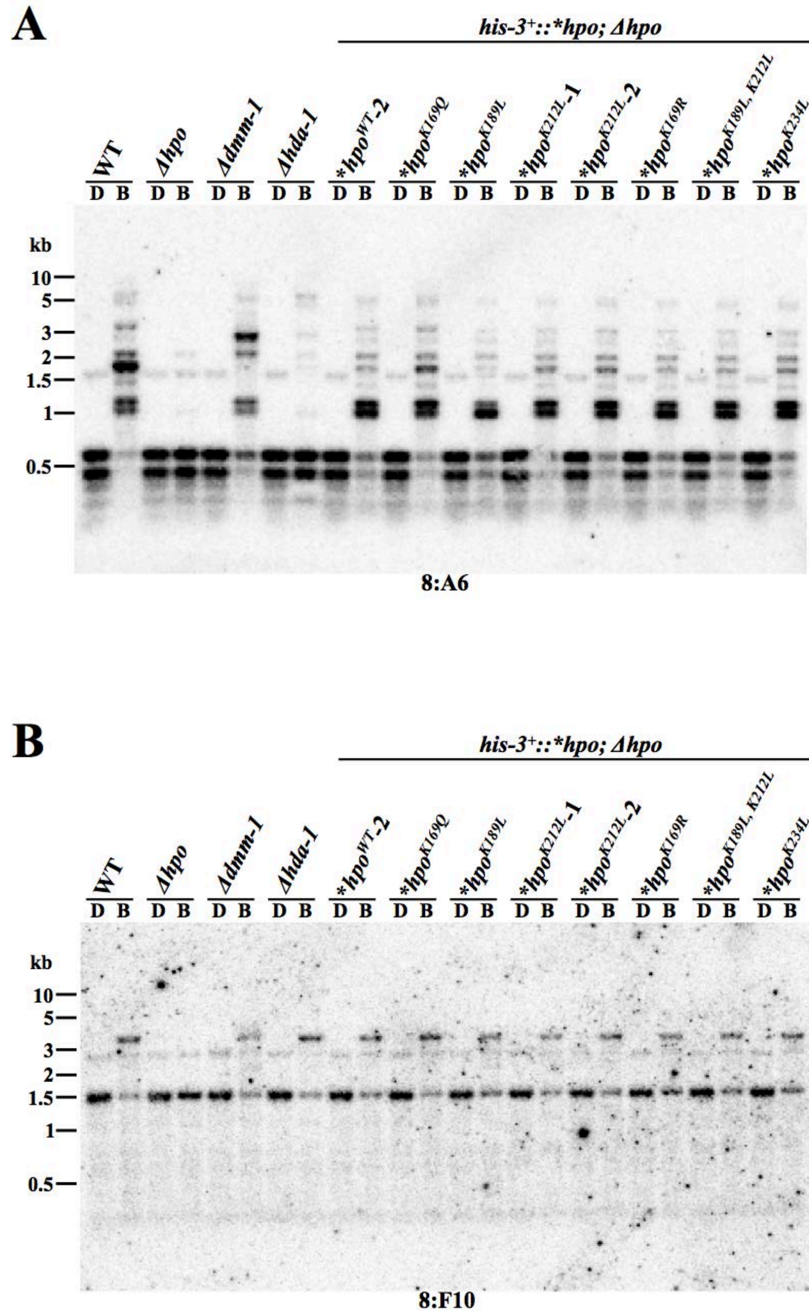

Figure 8: Decreased Silencing in PTM-Site Mutants is Not Associated with Consistent Changes in DNA Methylation at Known Heterochromatin Domains

Genomic DNA from N3752, N5580, N3064, N3610, N6291, N6292, N6293, N6294, K212L-2, N6296, N6297, and K234L were digested with 5mC-insensitive *DpnII* (D) or its 5mC-sensitive isoschizomer *BfuCI* (B), then analyzed by Southern hybridization with probes for the **A)** 8:A6, and **B)** 8:F10 heterochromatin domains. hpo^{WT}-2 - hpo^{K234L} denote substitutions in *phpo::hpo-10xGly-HAT-3xFLAG* at the *his-3* locus in a *Δhpo* background.

Decreased Silencing in PTM-Site Mutants is Not Accompanied by Consistent Hypermethylation of Centromeres

Although no decrease in DNA methylation at 8:A6 was detected, we reasoned that partial defects in HCHC activity might disrupt centromeric silencing without significantly affecting 8:A6. To explore this possibility, we investigated cytosine methylation of the centromeres, which is greater in knockouts of HCHC. Although the exact mechanism has not been conclusively established, it has been proposed that centromeric hypermethylation in HCHC-deficient strains is an indirect effect mediated by changes in chromatin conformation[21]. To test whether the same effect was present in the PTM-site mutants, I examined DNA methylation at two centric loci in linkage group 7 (LGVII).

I detected hypermethylation of *CenVIIR* in the *Δhda-1* control, but this effect seemed weaker than has been previously reported in literature, and was visible only as a faint smear extending above the major bands (Fig. 9A)[21]. As with 8:F10, no appreciable differences in methylation were apparent between WT and *hpo*^{WT}-2, indicating that the *hpo* construct essentially rescued DNA methylation at *CenVIIR* from the *Δhpo* phenotype. *Δdmm-1* seemed to produce modest hypomethylation at this locus, reflecting its general antagonism with HCHC and its tendency to decrease methylation at the core of heterochromatin domains (Honda *et al,* unpublished)[21,23]. With the other mutants, no definitive changes were seen, but a few subtle differences were observed. In *hpo*^{K212L}-1 and *hpo*$^{K189L,\ K212L}$, a slight decrease in the smallest fragment paired with barely noticeable increases in the largest two fragments (~ 1.5 kb and 2.2 kb) could indicate minor hypermethylation. However, the absence of this effect in *hpo*^{K212L}-2 shows that

this change is inconsistent and may be caused by other factors. *hpo*^{K234L} appears to have a slightly more noticeable decrease in methylation at this locus, but care must be taken to avoid over-interpretation of this difference, which is relatively small and has not been reproduced.

CenVIIL also sported only minor differences rather than consistent hypermethylation in the mutants (Fig. 9B). Here, the absence of *hda-1* produced much greater hypermethylation than at *CenVIIR*, which indicates that defects in HCHC should be easier to detect at this locus. The loss of *dmm-1* produced little change in methylation. Rescue of DNA methylation by the *hpo*^{WT}-2 appears to be slightly incomplete, considering that the larger fragments (~ 3 kb and 4.5 kb) are somewhat fainter than in WT. Perhaps the only notable changes in DNA methylation at this locus were found in *hpo*^{K212L}-2 and *hpo*^{K234L}, which presented some degree of hypermethylation relative to *hpo*^{WT}-2 and possibly even to the endogenous WT. This is evident in the increased ratio of the middle fragment (~ 3 kb) to the lower fragment (~ 2.2 kb). Again, the discrepancy between the sister *hpo*^{K212L} strains demands caution in interpreting these changes, which cannot be directly attributed to the substitutions in *hpo*. Furthermore, *hpo*^{K234L} exhibited equally significant hypomethylation at *CenVIIR*, so limited hypermethylation at this locus is not necessarily representative of more general patterns of centromeric DNA methylation.

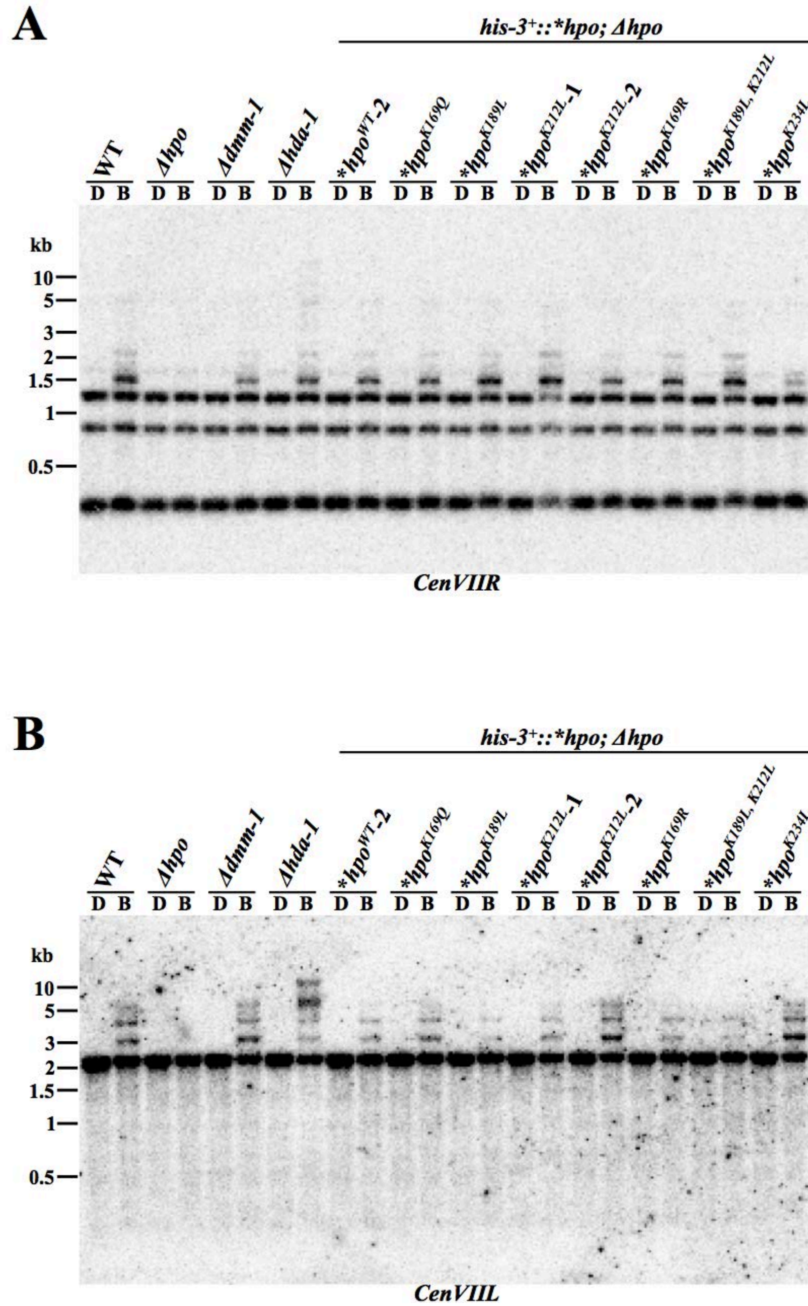

Figure 9: DNA Methylation in the Pericentric Heterochromatin of PTM-Site Mutants

Genomic DNA from N3752, N5580, N3064, N3610, N6291, N6292, N6293, N6294, K212L-2, N6296, N6297, and K234L were digested with 5mC-insensitive *DpnII* (D) or its 5mC-sensitive isoschizomer *BfuCI* (B), then analyzed by Southern hybridization with probes for the **A)** *CenVIIR*, and **B)** *CenVIIL* pericentric regions. *hpo*^{WT}-2 - *hpo*^{K234L} denote substitutions in *phpo::hpo-10xGly-HAT-3xFLAG* at the *his-3* locus in a *Δhpo* background.

Normal Containment of DNA Methylation at Boundaries of Heterochromatin Domains in PTM-Site Mutants

In accordance with our objectives, we also explored the effect of PTM-site substitutions on the functionality of the DMM complex. The primary consequence of defective DMM is the spreading of heterochromatin (as measured by its epigenetic hallmarks) beyond normal heterochromatin domains and into adjacent euchromatin. We assessed DNA methylation at a flanking region of the 8:G3 heterochromatin domain. This euchromatic border area is known to be hypermethylated in DMM knockouts but unmethylated in WT (Fig. 10A)[23]. This was independently verified by Southern analysis, revealing extensive hypermethylation in the *Δdmm-1* strain. However, the 8:G3 boundary remained unmethylated in the other controls and in the *hpo* mutants. While this shows that none of the mutant strains have defects only in DMM, deletion of HCHC components is known to prevent methylation spreading in a *Δdmm-1* background (Honda *et al*, unpublished).

Lastly, I also probed for the euchromatic cyclosporin A resistance gene (*csr-1*) as a control to check for completion of the digestion reactions (Fig. 10B). Sharp, well-resolved bands of equal size, position, and intensity in the *DpnII* and *BfuCI* products of each strain confirmed apparent full digestion of genomic DNA and equal loading of samples.

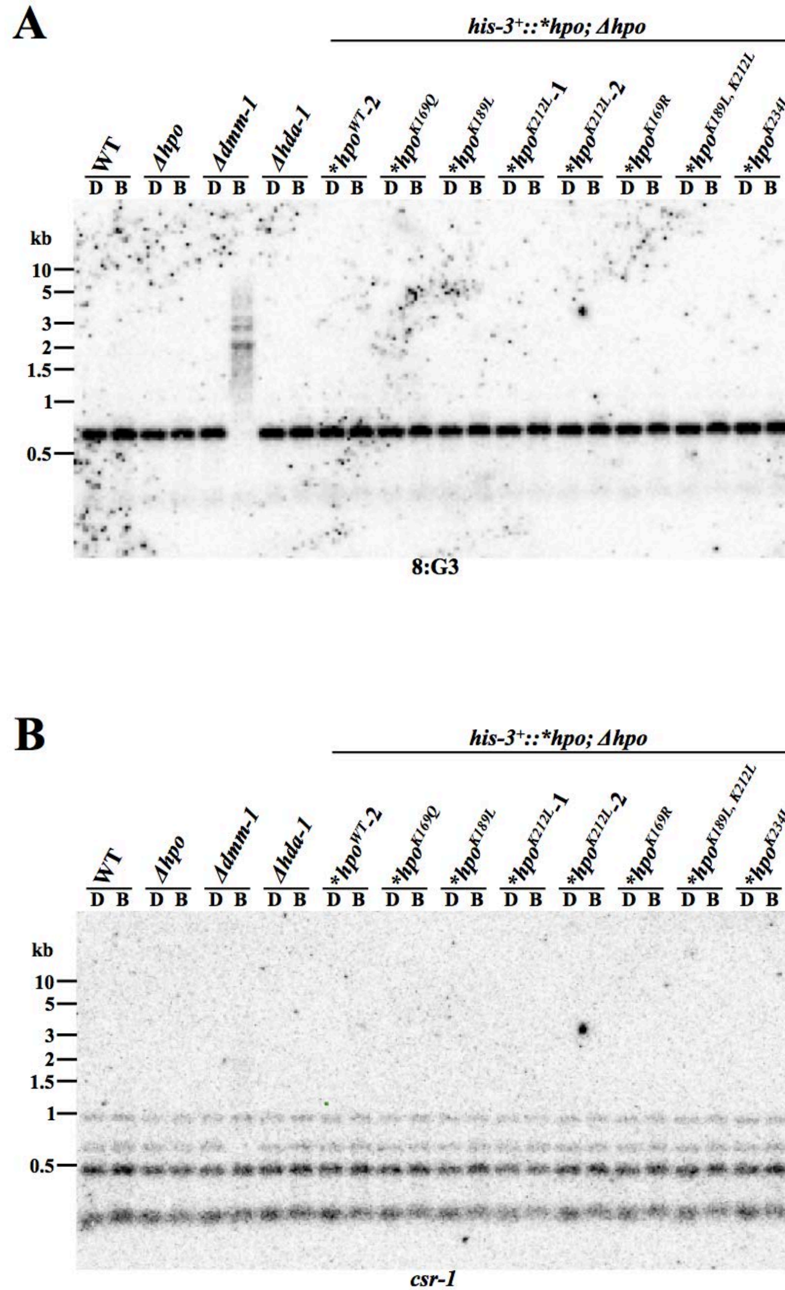

Figure 10: Control of Heterochromatin Spreading is Not Disrupted in Strains With Substitutions at Particular PTM-Sites of HP1

Genomic DNA from N3752, N5580, N3064, N3610, N6291, N6292, N6293, N6294, K212L-2, N6296, N6297, and K234L were digested with 5mC-sensitive *DpnII* (D) or its 5mC-sensitive isoschizomer *BfuCI* (B), then analyzed by Southern hybridization with probes for **A)** the euchromatic flank of the 8:G3 heterochromatin domain, and **B)** the *csr-1* euchromatin control region. hpo^{WT}-2 - hpo^{K234L} denote substitutions in *phpo::hpo-10xGly-HAT-3xFLAG* at the *his-3* locus in a *Δhpo* background.

Discussion

Specific Requirement of Certain HP1 PTMs for Centromeric Silencing

I found a substantial loss of centromeric silencing in strains where the K169, K189, K212, or K234 PTM-sites of HP1 were substituted with other amino acids to prevent (or mimic) modification. While silencing was decreased by mutations at each one of these sites, a corresponding decrease in other tested HP1 functions was not detected. Rescue of DNA methylation at known heterochromatin domains shows that loss of the targeted PTMs does not significantly affect the recruitment of DIM-2 or more general HP1 functions like dimerization and localization to H3K9me3. Although the results are not yet conclusive, they do support our initial hypothesis that PTMs confer functional specialization to HP1 by modulating its interactions with other proteins. If these findings are replicated and verified in further experiments, they may provide the first evidence that PTMs selectively facilitate distinct HP1 functions in Neurospora. However, the mechanism by which these PTMs participate in centromeric silencing is currently unclear. In the following sections, I propose two feasible mechanisms to be explored in future work.

Putative Dependency of Mi-2 Activity on PTMs in the Chromoshadow Domain

HCHC plays a documented role in centromeric silencing, but new work has found that the ATP-dependent chromatin remodeler Mi-2 is also recruited by HP1 and required for centromeric silencing in Neurospora (Honda *et al,* manuscript in preparation). Accordingly, the partial relief of *CenVIR::bar* expression observed in the PTM mutants could have resulted from defects in the recruitment of either Mi-2 or

HCHC. However, strains deficient in HCHC reliably exhibit hypermethylation at centromeric loci and loss of DNA methylation at some other heterochromatin domains, including 8:A6[21]. These effects were clearly detected by Southern analysis of the *Δhda-1* control, but are not visible in the PTM-site mutants with faulty silencing. The pattern of DNA methylation in these mutants is inconsistent with a mechanism that only affects HCHC activity.

By comparison, knockouts of *mi-2* display normal levels of DNA methylation at centromeric loci, and only a minor decrease of DNA methylation at 8:A6, rather than a total loss of it. In addition, *Δmi-2* strains appear to have slightly lower expression of *CenVIR::bar* than those with loss of HCHC components (Honda *et al,* manuscript in preparation), possibly explaining why the PTM-site mutants were less resistant to Basta than the *Δhpo* and *Δhda-1* controls. Because the Mi-2 findings were brought to our attention after the completion of the experiments in this study, *Δmi-2* Neurospora was not included as an experimental control for direct comparison with the mutants. Despite this omission, the observed phenotypes of the PTM-site mutants are in good agreement with the reported phenotypes of *mi-2* knockouts, although the incomplete relief of *CenVIR::bar* expression suggests the loss of Mi-2 activity may be only partial. Furthermore, the forthcoming publication by Honda *et al* found that HP1 interacts with Mi-2 through its chromoshadow domain, which contains 2 of the 4 sites investigated (K234 and K212). The other two PTM sites (K189 and K169) are just outside the chromoshadow domain in the adjacent periphery of the hinge region, where they could plausibly exert an effect on the interaction (Fig. 3). Together, the results indicate that PTMs at the aforementioned sites participate in the interaction between HP1 and Mi-2,

and that the absence of these PTMs disrupts the function of the complex between these proteins.

Defects in HCHC Activity

Although a defect in Mi-2 activity is sufficient to explain the phenotype observed in the PTM-site mutants, other explanations are also possible, and cannot yet be excluded from consideration. In particular, it is possible that the decrease in centromeric silencing was caused by a defect in HCHC activity that did not produce the characteristic changes in DNA methylation. Although this has not been observed in past studies, there are at least two feasible ways by which relatively normal levels of DNA methylation could have been maintained in strains with HCHC deficiency[21].

Tiered Phenotype Effects in Strains with Partial Loss of HCHC

Defective HCHC causes loss of centromeric silencing, centromeric hypermethylation, and hypomethylation at some heterochromatin domains (8:A6), but these effects may be induced at different thresholds. Relatively minor defects in the recruitment or activation of HCHC could cause detectable decreases in silencing like those seen in the PTM-site mutants, but changes in DNA methylation might occur only when HCHC is severely defective. The mechanism of centromeric hypermethylation in HCHC-deficient strains remains undetermined, but the leading hypothesis contends that histone hyperacetylation causes a relaxation of chromatin conformation and thereby increases its accessibility to the DIM-2 DNA methyltransferase[21]. The PTM-site mutants have only partial loss of centromeric silencing, indicating that some degree of HCHC function is preserved. Perhaps the partial loss of HCHC caused a change in the

conformation of centromeric chromatin that was just sufficient to express *CenVIR::bar* at modest levels, but not enough to substantially increase its accessibility to DIM-2. If a similar mechanism is responsible for other changes in the methylation profile of HCHC knockouts, then these changes may be similarly absent in strains where defects in HCHC are only moderate.

Concomitant Antagonistic Defects in HCHC and DMM

Another way in which typical indicators of HCHC function could possibly be concealed is through antagonism between the HCHC complex and the DMM complex. While cytosine methylation spreading was not detected at the 8:G3 heterochromatin boundary in any of the PTM mutants, loss of HCHC has been shown to prevent heterochromatin spreading in a *Δdmm-1* background (Honda *et al*, unpublished). If the examined PTMs are required for both the function of the DMM complex and the HCHC complex, the loss of DMM function in the PTM-site mutants would not induce cytosine methylation spreading at 8:G3, because spreading cannot occur in the absence of HCHC.

In this case, the effects of DMM deficiency on the HCHC phenotype must also be considered. While such interactions have not been thoroughly explored by experimental means at the time of this writing, the antagonistic functions of HCHC and DMM may be a two-way relationship rather than a unidirectional pathway. Such a scenario can easily be imagined through a comparison of their disparate phenotypes. Whereas HCHC knockouts cause centromeric hypermethylation, DMM knockouts tend to cause hypomethylation at the interior of centromeres, although this effect is not clearly visible at the probed centromeric loci (Fig. 9)[21,23]. While *Δhda-1* was seen to

cause major hypomethylation at 8:A6, *Δdmm-1* produced marked hypermethylation (Fig. 8A). For many regions of the genome, including those analyzed by Southern blotting in this study, it is feasible that these antagonistic effects may roughly cancel out, perhaps contributing to phenotypic instability and high variation between progeny. Because of this, it is entirely possible that the PTM mutants have dual reductions in the activity of both complexes.

Other Possible Causes of Mutant Phenotypes

Rather than promoting the recruitment of HP1 binding partners, the PTMs under investigation could confer functional specialization by inhibiting specific interactions with other proteins. Such a mechanism could conceivably be responsible for the preferential localization of the DMM complex to the periphery of heterochromatin domains, preventing it from antagonizing heterochromatin establishment at the interior of centromeres. Eliminating such a modification might cause a loss of centromeric silencing like that detected in this study, although the effects on DNA methylation may or may not match the observed phenotypes.

Alternatively, it is possible that currently unknown chromatin-associated factors or signaling proteins interact with HP1 to facilitate other important functions, which may feed back into the known heterochromatin pathways. This possibility is supported by the recent discovery of Mi-2's functionally significant interaction with HP1 (Honda *et al*, unpublished). Similar interactions with other chromatin modifiers or remodelers may be awaiting discovery. Equally suggestive is the extensive modification of HP1 by unidentified enzymes in response to unknown signals. It is both plausible and likely that the modifiers of HP1 are influenced by PTMs, which may serve as a dynamic interface

where information from multiple pathways is stored. Substitutions at a given PTM-site may affect the modification of other sites. As an example, the recruitment of Mi-2 might be directly controlled solely by acetylation of K169. However, the lysine acetyl-transferase responsible for acetylation of this site might be active only if K189, K212, and K234 are formylated. In this way, a particular function of HP1 may be directly mediated by only a single PTM, but indirectly regulated by a larger network of PTMs with linked functions. Characterizing PTM mutants with substitutions at multiple sites will help to address this possibility.

It is also worth noting that strains with hpo^{K169R} and hpo^{K169Q} both exhibited a similarly substantial decrease in silencing. If arginine substitution mimics a loss of acetylation and glutamine substitution mimics constitutive acetylation, these substitutions should have opposite effects, and normal silencing should be seen in one of the two strains. The deviation from this expectation likely indicates that glutamine substitution did not truly mimic constitutive acetylation. Even though the charge of this residue is similar to that of acetyl-lysine, the structure may be sufficiently different to disrupt any interactions that depend on lysine acetylation.

Many of the proposed mechanisms are somewhat speculative, but the evidence gathered in this study is not sufficient to disprove them. The proposed dependence of Mi-2 recruitment on PTMs in the chromoshadow domain is by far the simplest, most direct, and most likely explanation for the results of this study. However, the large number of alternative plausible alternative mechanisms demonstrates the need for further study to elucidate the true nature of this system.

Future Directions

The largest limitation of this project as it currently stands is the lack of multiple replicates for each of the mutant strains. Previous studies have established that even clonally related strains propagated from individual conidia of a single parent have stable differences in their levels of gene expression and DNA methylation[51]. Background phenotypic variation between strains introduced considerable uncertainty into the results. While this does not undermine the significance of the decrease in centromeric silencing observed in all of the mutants, it does make it much more difficult to compare subtle differences between them. To eliminate any differences caused by ectopic expression at the *his-3* locus, each construct will be inserted at the endogenous *hpo* locus, and multiple transformants carrying each mutation will be characterized. This will make it possible to overcome the variation and to establish a representative phenotype for each mutation. In addition, I will characterize the remaining mutant strains that have not yet been tested.

Future research will also explore the possible mechanisms responsible for these phenotype effects. To ascertain whether the observed defects in silencing are caused by decreased activity of Mi-2 or HCHC, the endogenous *hpo* mutants will be crossed into a *Δdmm-1* background. If the *hpo* substitutions inhibit HCHC activity, they should eliminate DNA methylation spreading at 8:G3 in a *Δdmm-1* background. If a reduction in Mi-2 activity is responsible for the silencing defects, spreading at 8:G3 will persist in the *Δdmm-1* PTM mutant progeny.

To verify the results of this experiment, I will test heterochromatin silencing at transposon relics, which requires HCHC but not Mi-2 (Honda *et al,* manuscript in

preparation). This will be tested by assessing the silencing of a *bar* marker that has been inserted between RIP'd duplicate copies of *his-3* (*his-3*^{RIP}*::bar*). Progeny carrying this marker and the desired substitutions in endogenous *hpo* will be isolated from an appropriate cross and spot tested on Basta. Basta resistance would suggest loss of HCHC activity, and silencing would indicate loss of Mi-2 function. Knockout of *mi-2* is also known to cause DNA methylation spreading at characteristic sites that are unmethylated in WT, *Δdmm-1*, or *Δhda-1*. Probing for these regions in a methylation-sensitive Southern analysis will provide additional support for the results of the other assays.

In addition, I will use co-immunoprecipitation to directly measure changes in affinity between the mutated HP1 protein and Mi-2 or CDP-2, which is the direct interacting partner of HP1 in the HCHC complex[21]. If the Mi-2 mechanism is correct, I should detect decreased affinity of the mutagenized HP1 protein for Mi-2 without a corresponding decrease in its affinity for CDP-2. Conversely, a selective decrease in the affinity for CDP-2 would support a mechanism mediated by HCHC. It is also possible that the substitutions inhibit the activity of Mi-2 or HCHC without disrupting their recruitment by HP1, in which case no decrease in affinity would be expected. In conjunction with the experiments mentioned above, this method will expose differences between the existing hypotheses and provide more conclusive evidence for the mechanism of PTM-dependent centromeric silencing.

Another question I aim to answer in future work is whether mutation of multiple sites at the same time will lead to different phenotypes. While centromeric silencing, and possibly the recruitment of Mi-2, can be regulated by a subset of individual HP1

modifications, other interactions might be regulated by specific combinations of PTMs that are not active individually. To account for this possibility, we will use antibodies for epitope-tagged Mi-2, CDP-2, DMM-1, and DIM-2 to pull down each of the known HP1 complexes by CoIP. We will then affinity purify HP1 from each of these individual complexes and identify the characteristic PTM "fingerprints" by mass-spectrometry. To eliminate the need for affinity tags and the interference they may introduce, I am currently working to purify a polyclonal rabbit antibody against HP1 for affinity purification of the native untagged protein. The findings of this study suggest that PTMs at K169, K189, K212, and K234 may be preferentially found in HP1 purified from the Mi-2 complex. Such a result would demonstrate that PTMs are not only capable of regulating HP1 interactions, but that they are regularly used for this purpose in WT Neurospora. In addition, combinations of other differentially incorporated PTMs might also be identified in other complexes.

Conclusion

In furtherance of our initial hypothesis, this study successfully identified a subset of HP1 PTM sites that are specifically required for centromeric silencing, but not for other HP1-dependent functions. The existence of an "HP1 subcode" has long been speculated, but this study provides the first evidence for such a code in Neurospora[25,26]. The findings show that PTMs can be used to facilitate functional diversification in HP1, enabling local control of its activity. While a number of less direct explanations cannot be ruled out at this time, the observed phenotypes are entirely consistent with a specific decrease in the recruitment of Mi-2. Future work will seek to replicate these results with mutant *hpo* expression at the endogenous locus and to conclusively determine the exact

mechanism of these effects. Further research will also be aimed at identifying PTMs with different frequencies in distinct HP1 complexes, which would be likely regulators of HP1 function.

A comprehensive understanding of PTM effects on HP1 function will help to elucidate the relationship between the major chromatin modifier complexes. This understanding could lay the foundation for the identification and characterization of the upstream enzymes that modify HP1, and for research into the regulation of their own activity. This study brings us closer to understanding how these regulatory pathways fit into a larger network of epigenetic regulation that connects dynamic states of chromatin to shifting environmental conditions and perhaps to signaling between cells. Ultimately, this knowledge could help to identify targets for drugs that treat the abnormal patterns of DNA methylation and other epigenetic markers in diseases like cancer and autism.

Supplementary Information

Supplementary Table S1. Primers used in this study

Primer	Sequence
Site-directed mutagenesis	
hpo^{K104R}	
#4549	5'-GGGTTACGAGAAAAGGTCTGACCAGACCTGG-3'
#4550	5'-CCAGGTCTGGTCAGACCTTTTCTCGTAACCC-3'
hpo^{K104Q}	
#4551	5'-GGGTTACGAGAAACAGTCTGACCAGACCTGG-3'
#4552	5'-CCAGGTCTGGTCAGACTGTTTCTCGTAACCC-3'
hpo^{K124L}	
#4553	5'-GCGTCGGAGAGGCTCCTGGAGTACTTTACCAAG-3'
#4554	5'-CTTGGTAAAGTACTCCAGGAGCCTCTCCGACGC-3'
hpo^{K124R}	
#4555	5'-GTCGGAGAGGCTCAGGGAGTACTTTACCAAG-3'
#4556	5'-CTTGGTAAAGTACTCCCTGAGCCTCTCCGAC-3'
hpo^{K124Q}	
#4557	5'-GTCGGAGAGGCTCCAGGAGTACTTTACCAAG-3'
#4558	5'-CTTGGTAAAGTACTCCTGGAGCCTCTCCGAC-3'
hpo^{Y126F}	
#4541	5'-GAGAGGCTCAAGGAGTTCTTTACCAAGATTGGCG-3'
#4542	5'-CGCCAATCTTGGTAAAGAACTCCTTGAGCCTCTC-3'
hpo^{T128A}	
#4543	5'-CTCAAGGAGTACTTTGCCAAGATTGGCGGTCG-3'
#4544	5'-CGACCGCCAATCTTGGCAAAGTACTCCTTGAG-3'
hpo^{K129L}	
#4559	5'-CTCAAGGAGTACTTTACCCTGATTGGCGGTCGAGAG-3'
#4560	5'-CTCTCGACCGCCAATCAGGGTAAAGTACTCCTTGAG-3'
hpo^{K169R}	
#4561	5'-CAAACGGTCGAGGAGGAACGGCGACC-3'
#4562	5'-GGTCGCCGTTCCTCCTCGACCGTTTG-3'
hpo^{K169Q}	
#4563	5'-CAAACGGTCGAGGCAGAACGGCGACC-3'
#4564	5'-GGTCGCCGTTCTGCCTCGACCGTTTG-3'

hpo^{K189L}
#4565 5'-CGCTGCCTGGCTGCCCCCAGCGGG-3'
#4566 5'-CCCGCTGGGGGCAGCCAGGCAGCG-3'

hpo^{K212L}
#4567 5'-GAAGATGAGGACACACACCTGCTCATGGTTTATCTGACG-3'
#4568 5'-CGTCAGATAAACCATGAGCAGGTGTGTGTCCTCATCTTC-3'

hpo^{Y233F}
#4545 5'-CAACTGACGTCATCTTCAAGAGGTGCCCCC-3'
#4546 5'-GGGGGCACCTCTTGAAGATGACGTCAGTTG-3'

hpo^{K234L}
#4764 5'-GACGTCATCTACCTGAGGTGCCCCCAAAAGGTAC-3'
#4765 5'-GTACCTTTTGGGGGCACCTCAGGTAGATGACGTC-3'

hpo^{S263A}
#4547 5'-GGATCGCGAGGGCGCCGTCTCGCAACAATTC-3'
#4548 5'-GAATTGTTGCGAGACGGCGCCCTCGCGATCC-3'

Sanger sequencing
4539_HP1 (-82)F
#4539 5'-ACTGCAACGCCCCCCGTTCC-3'

HP1 (+450)_seq_Fwd
- 5'-CTAATATAGTATTGCTCATCACAG-3'

Southern probes
8:A6 (303 bp)
#1823 5’-GGATGGCGGATCCTCAAAAATA-3’
#1824 5’-TAACCGCCGCTTTTTAAAATTAGGA-3’

8:F10 (317 bp)
#1827 5’-GTAACGCAAATTCTAAAATTGCAATAC-3’
#1828 5’-CTTAGTAATTAATTTAATACGTGCGCC-3’

CenVIIR (294 bp)
#2541 5’-GGAGGTATAGAGGTACTAGGAG-3’
#2562 5’-CTTATTAAAGGTGCCCAGATATAATAGTAG-3’

CenVIIL (261 bp)
#2539 5’-CTTCTACTAGACCTAAGGGAGG-3’
#2550 5’-CGTCTTTGATAGTCGGGGATAAGG-3’

8:G3 (765 bp)
#1845 5'-CGATTCCTCGGACCTCGATC-3'
#1846 5'-TCATGCCGCTTCGTGATCTG-3'

3' *csr-1* flanking region (1049 bp)
#4654 5'-AACACCTCCGTCGCCATAAACTCC-3'
#4722 5'-TGGATTTCCTGCGCTGCACAC-3'

Supplementary Table S2. *Neurospora crassa* strains used in this study

Strain	Genotype	Reference
N3064	*mat A his-3; Δdmm-1::hph*$^{+}$	Honda *et al* (2010)
N3610	*mat A his-3; Δhda-1::hph*$^{+}$	FGSC #12003
N3752	*mat A*	2489
N4890	*mat A his-3*; *CenVIR::bar trp-2*	Honda *et al* (2012)
N4891	*mat a his-3*; *CenVIR::bar hpo*	Honda *et al* (2012)
N4906	*mat a his-3*; *CenVIR::bar trp-2; Δhda-1::hph*$^{+}$	Honda *et al* (2012)
N5580	*mat a his-3; Δhpo::hph*$^{+}$	Ormsby *et al* (unpublished)
N5889	*mat a his-3*$^{+}$*::phpo::hpo*^{WT}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	Ormsby *et al* (unpublished)
N5891	*mat a his-3*$^{+}$*::phpo::hpo*^{T108A}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	Ormsby *et al* (unpublished)
N6291	*mat ? his-3*$^{+}$*::phpo::hpo*^{WT}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
N6292	*mat ? his-3*$^{+}$*::phpo::hpo*^{K169Q}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
N6293	*mat ? his-3*$^{+}$*::phpo::hpo*^{K189L}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
N6294	*mat ? his-3*$^{+}$*::phpo::hpo*^{K212L}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
N6296	*mat ? his-3*$^{+}$*::phpo::hpo*^{K169R}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
N6297	*mat ? his-3*$^{+}$*::phpo::hpo*$^{K189L, K212L}$*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
K212L-2	*mat ? his-3*$^{+}$*::phpo::hpo*^{K212L}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study
K234L	*mat ? his-3*$^{+}$*::phpo::hpo*^{K234L}*-10xGly-HAT-3xFLAG; CenVIR::bar trp-2 Δhpo::hph*$^{+}$	This study

Supplementary Table S3. Recipes used in this study

Mixture	Composition
Bacterial growth media	
LB broth	1% tryptone, 1% NaCl, 0.5% yeast extract
LB agar	1.5% agar, 1% tryptone, 1% NaCl, 0.5% yeast extract
SOC medium	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose
Neurospora growth media	
Vogel's trace element solution	5 g $Na_3Citrate \cdot 1\ H_2O$, 5 g $ZnSO_4 \cdot 7\ H_2O$, 1 g $(NH_4)_2Fe(SO_4)_2 \cdot 6\ H_2O$, 250 mg $CuSO_4 \cdot 5\ H_2O$, 50 mg $MnSO_4 \cdot 1\ H_2O$, 50 mg H_3BO_3, 50 mg $Na_2MoO_4 \cdot 2\ H_2O$, 100 ml final volume in water
Biotin solution	50% ethanol, 50 μg/ml biotin
50x Vogel's medium N	150 g $Na_3Citrate \cdot 5\ H_2O$, 250 g KH_2PO_4, 100 g NH_4NO_3, 10 g $MgSO_4 \cdot 7\ H_2O$, 5 g $CaCl_2 \cdot 2\ H_2O$, 5mL biotin solution (50 μg/ml), 5 ml trace element solution, 1 liter final volume in water
50x Synthetic crossing medium (SC)	250 g KH_2PO_4, 150 g $Na_3Citrate \cdot 5\ H_2O$, 10 g NH_4NO_3, 10 g $MgSO_4 \cdot 7\ H_2O$, 5 g $CaCl_2 \cdot 2\ H_2O$, 5mL biotin solution (50 μg/ml), 5 ml trace element solution, 1 liter final volume in water
FGS Vogel's agar (Colonial growth)	1.5% agar, 1x Vogel's medium N, 2% sorbose, 0.05% fructose, 0.05% glucose
SC agar (crossing medium)	1.5% agar, 1x SC medium, 2% sorbose, 0.05% fructose, 0.05% glucose
Basta medium	1.5% agar, 1x nitrogen-free Vogel's medium (no NH_4NO_3), 2% sorbose, 0.5% proline, 0.05% fructose, 0.05% glucose, desired concentration of Basta
Western blotting buffers	
Lysis buffer	10% glycerol, 150 mM NaCl, 50 mM HEPES pH 7.5, 10 mM EDTA, 0.02% NP40, EDTA-Free Complete Mini Protease Inhibitor Cocktail (Roche, 1 tablet per 10 ml).

2x Laemmli buffer	20% glycerol, 4% SDS, 125 mM Tris (pH 6.8), 0.02% bromophenol blue
TBS-T	10 mM Tris (pH 7.5), 150 mM NaCl, 0.05% Tween 20
Concentrations of Drugs and Supplements	
Ampicillin	100 µg/ml (1x) diluted from 100 mg/ml stock (1000x)
Hygromycin	2 µg/ml (1x) diluted from 1 mg/ml stock (500x)
Basta	8 mg/ml (1x) diluted from 400 mg/ml stock (50x)
Histidine	500 µg/ml (1x) diluted from 25 mg/ml stock (50x)
Tryptophan	200 µg/ml (1x) diluted from 10 mg/ml stock (50x)

Glossary

3' and 5': The two opposite ends of a DNA stand, which are used to establish directionality. On the coding strand of DNA, the start of a gene will be towards the 5' end relative to the end of a gene, which is closer to the 3' end.

Acetylation: The covalent addition of an acetyl group to a substrate. An acetyl group contains two carbons, one of which is a methyl group, and the other of which is bound to the substrate and double bonded to oxygen. This addition can change the physical and chemical properties of the target molecule.

Activation: This can refer to an increase in the level of gene expression, or an increase in the activity of the protein product.

Affinity: The strength of intermolecular interactions between a protein and its binding partner.

Affinity tag: A polypeptide sequence with high affinity for a specific ligand or antibody. Proteins are expressed with affinity tags so they can be easily purified with immobilized ligand or visualized with labeled antibodies.

Antibody: A protein generated by immune cells that binds a target protein or affinity tag with high specificity. By fluorescently or radioactively labeling and antibody, it is possible to visualize the target protein.

Basta: An herbicide, also known as glufosinate, that is lethal to wild type Neurospora. Resistance to Basta is conferred by the *bar* gene.

Centromere: The central part of a chromosome that links identical chromosome copies (sister chromatids). The centromere is composed of heterochromatin.

CenVIR::bar: A Basta resistance gene that has been inserted into the pericentric region of the right arm of chromosome six.

ChIP-Seq: Chromatin immunoprecipitation sequencing, a method used to map interactions between DNA and a protein of interest. ChIP-Seq quantifies the level of protein binding across the genome.

Chromatin: The complex of DNA, histone proteins, and other DNA-associated factors in the nucleus.

Chromodomain: A conserved protein domain that mediates binding to chromatin. The HP1 chromodomain binds H3K9me3.

Chromoshadow domain: The structural domain of HP1 that mediates dimerization and interactions with other proteins such as DIM-2. HP1 functions as a homodimer.

Chromosome: Large bundles of chromatin inside the nucleus. The genomes of most eukaryotes are divided into a number of chromosomes. The chromosome structure helps to preserve the genome and to facilitate segregation during mitosis. Neurospora has seven chromosomes.

Cloning: The process by which DNA from multiple sources can be combined in a single DNA molecule. Inserting a gene or DNA sequence into a bacterial plasmid is a common form of cloning. If recombinant DNA is taken through further stages of cloning, it is often referred to as recloning.

Complex: Any group of proteins or other macromolecules that physically interact to form a single structure.

Conidia: Asexual, haploid vegetative spores.

DCDC: A five-member complex containing DIM-5, DIM-7, DIM-9, CUL4, and DDB1. DCDC generates H3K9me3 through the methyltransferase activity of DIM-5 after the complex localizes to A:T-rich DNA, possibly through DIM-7.

DIM (Defective in DNA Methylation): This can refer to a mutant strain of Neurospora, a gene required for normal DNA methylation, or the protein product of a gene required for DNA methylation.

DIM-2: A DNA methyltransferase responsible for all known DNA methylation in Neurospora. HP1 is required for the proper localization of DIM-2

DMM complex: The DNA methylation modulator complex containing HP1, DMM-1, and DMM-2. This complex prevents the aberrant spreading of DNA and H3K9 methylation beyond the boundaries of heterochromatic regions. The exact mechanism is not known, but it has been suggested that DMM-1 may act as a histone demethylase.

DNA methylation: An epigenetic marker associated with silencing and heterochromatin in eukaryotes. Methylated DNA is characterized by the presence of a methyl group attached to cytosine at the carbon-5 position.

Electroporation: The use of a small electric current to make cells more permeable, allowing DNA to enter. Electroporation is a process used in the transformation of cells.

Epigenetics: The study of heritable or semi-heritable changes in gene expression that are not caused by the DNA sequence alone.

Euchromatin: Loosely packed, transcriptionally active chromatin.

Eukaryote: An organism composed of cells with a nucleus and other membrane subcompartments.

Gel electrophoresis: The use of an electric current to drive macromelecules such as proteins and DNA through pores in a gel, causing them to separate by size and charge.

Gene expression: The rate at which a gene is transcribed and translated into a functional protein product. A gene cannot be active if it is not expressed.

Genome: The full DNA sequence of an organism.

H3K9: Lysine nine of histone H3. This amino acid can be trimethylated to give H3K9me3, which is a heterochromatin hallmark associated with silencing. It can also be acetylated to give H3K9Ac, which favors the formation of euchromatin and the activation of local genes.

HCHC complex: A protein complex containing HP1, chromodomain protein-2 (CDP-2), histone deacetylase-1 (HDA-1), and CHAP. This complex localizes to H3K9me3 and deacetylates adjacent H3K9. This process is essential for centromeric silencing.

Heterochromatin: Densely packed chromatin with minimal transcription. Heterochromatin performs many important functions involved in the structural integrity of chromosomes and in gene regulation.

his-3: One of the genes required for the synthesis histidine in *Neurospora*. The *his-3* locus is a common target for transformation.

Histone: A family of proteins that interact with DNA to form chromatin. Histones H2A, H2B, H3, and H4 form the protein core of the nucleosome. Modifications of histone H3 play an important role in regulating gene expression.

Homodimer: A protein complex containing two identical proteins.

Homology: Similarity in sequence, structure, or function between distinct proteins or DNA sequences. Sequence homology is used as a measure of relatedness, and sequences with high homology are said to be homologous. Homologous sequences can undergo recombination.

HP1: Heterochromatin protein one, a scaffolding protein that binds H3K9me3 and recruits the DIM-2 DNA methyltransferase, the HCHC complex, the DMM complex, or mi-2. HP1 is required for DNA methylation and the establishment of heterochromatin.

hpo: The gene for HP1 (heterochromatin protein one).

Hygromycin: An antibiotic that is lethal to wild type Neurospora. The *hph* gene confers resistance to hygromycin.

Knockout: An organism in which an existing gene was replaced (or disrupted) with an artificial sequence of DNA.

Ligand: A small molecule that binds to a protein. Immobilized ligands can be used to bind and purify their interacting protein.

Linearization: The use of a restriction enzyme to cut a plasmid, generating a linear segment of DNA.

Linker: A flexible polypeptide sequence that connects two globular subunits of a protein. The use of a linker between affinity tags and recombinant proteins minimizes steric crowding (prevents the groups from bumping into each other) that could otherwise cause the protein to misfold.

Locus: A specific site or region of the genome

Methylation: The covalent addition of a methyl group to a target molecule. A methyl group is composed of a single carbon atom bonded to three hydrogen atoms. DNA and many proteins are often methylated.

Nucleosome: The basic unit of chromatin. It consists of nuclear DNA coiled around an octamer protein complex containing two of each histone (H2A, H2B, H3, and H4).

PCR: The polymerase chain reaction replicates DNA templates. The number of DNA copies doubles with every round of PCR replication.

Pericentric: In close proximity to the centromere. Pericentric DNA is heterochromatic.

Plasmid: A DNA ring. Plasmids are common in many species of bacteria. Plasmids are smaller than chromosomes, and more easily passed between cells. This makes them useful for genetic manipulations such as cloning.

Primer: A short nucleotide sequence (typically 20-30 bp) used to initiate DNA replication. The complementarity of the primer dictates where replication will be initiated.

Probe: A short DNA sequence that is complementary to a target sequence. Probes are often radiolabeled and applied to Southern blots, which allows the visualization of specific fragments.

Promoter: A short DNA sequence that initiates transcription by recruiting transcription factors and RNA polymerase. Eukaryotes have a wide variety of diverse promoters. A gene's unique promoter is said to be its native promoter.

PTM: A post-translational modification, most often of HP1 in the context of this paper. A post-translational modification is any chemical group that is covalently attached to a protein after it is translated from mRNA by the ribosome. Phosphorylation, methylation, acetylation, and formylation are all PTMs.

Recombinant: A DNA segment containing genetic material from multiple sources.

Recombination: The exchange of information between two DNA molecules. Recombination is primarily used to trade segments between chromosome copies prior to meiosis, increasing genetic diversity. Recombination most often occurs between highly similar (homologous) sequences.

Restriction endonuclease: A group of enzymes that each cleave DNA at a specific sequence. Some restriction enzymes are methylation-sensitive, meaning they will only cut unmethylated targets.

RIP: Repeat-induced point mutations, which Neurospora uses to silence transposons and duplicated sequences. RIP causes these repetitive sequence elements to become A:T-rich targets for DCDC.

Scaffold protein: A protein that recruits other proteins and helps to facilitate their function, often by binding them in proximity to substrate with the proper orientation.

Silencing: A partial or total decrease in gene expression caused by a regulatory process. In epigenetics, silencing is associated with DNA methylation, H3K9me3, and heterochromatin establishment.

Site-directed mutagenesis: A variant of PCR that uses mismatched primers to generate specific mutations in copies of a DNA template.

Southern blotting: The transfer of DNA fragments from a gel onto a membrane. This allows the application of radiolabeled probes to visualize only the fragments of interest.

Telomere: The heterochromatic ends of chromosomes. Replication causes degradation at the free ends of DNA, but the structure of telomeres helps to preserve genetic stability.

Transcription: The process by which a DNA gene is used to make mRNA copies, which are later translated into a protein product. Transcription is the first step of gene expression. If a gene is expressed, it is also transcriptionally active.

Transformation: The introduction of foreign DNA into a cell.

Transposon: A DNA sequence that can change position within the genome. Many organisms, including Neurospora, silence transposons to prevent them from disrupting other genes.

Western blotting: The transfer of proteins from a gel onto a membrane. Western blotting allows specific proteins to be visualized by labeled antibodies.

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