

EPIGENETIC LANDSCAPE OF *NEUROSPORA CRASSA*: THE REGULATION AND
DYNAMICS OF H3K27ME3

by

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(Under the Direction of Zachary A. Lewis)

ABSTRACT

Chromatin, a complex of DNA and proteins, plays a vital role in regulating gene expression. This work focuses specifically on facultative heterochromatin, characterized by the tri-methylation of histone H3 at lysine 27 (H3K27me3), which serves as a molecular marker of transcriptionally silenced chromatin. This modification is deposited by the multi-subunit complex known as Polycomb Repressive Complex 2 (PRC2). To investigate the mechanisms governing the assembly and maintenance of facultative heterochromatin, we utilized the model fungus *Neurospora crassa*. Our findings reveal that a complex containing Histone Deacetylase-1 (HDA-1) in *N. crassa* plays a regulatory role in PRC2 activity. Notably, deletion of *hda-1* results in a global redistribution of H3K27 methylation from its typical PRC2-targeted domains to regions of constitutive heterochromatin, alongside observed genome instability linked to other histone modifications. Furthermore, we developed a reporter system that enables the separation of sequence-specific establishment of H3K27me3 from its maintenance across multiple generations. This research harnesses the unique attributes of *Neurospora* to enhance our understanding of the interactions between PRC2 and other factors in regulating the dynamics of facultative heterochromatin.

INDEX WORDS: Chromatin; Facultative heterochromatin; Constitutive heterochromatin;
Polycomb repressive complex 2; Histone Deacetylase; Assembly;
Maintenance

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BS, The University of Georgia, 2015

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2024

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December 2024

DEDICATION

To my mother, for all the sacrifices you have made to turn my dreams into reality. Your unwavering belief in me and your constant encouragement have been the foundation of my journey. You have always pushed me to strive for excellence, teaching me the value of hard work and resilience. I am deeply grateful for your love, support, and guidance, which have inspired me to reach for the best in everything I do. Thank you for being my greatest champion and for instilling in me the confidence to pursue my passions.

ACKNOWLEDGEMENTS

I would like to begin by acknowledging Allen and Trish Moore. Allen introduced me to science and recognized potential in me that I hadn't seen in myself. Together, they created an environment that made me believe I could become a scientist.

Next, I want to express my gratitude to Michelle Zadie. She exemplified what it means to be a graduate student and showed me how to thrive in graduate school. Her encouragement to apply for programs and her unwavering support made her the best mentor I could ask for. I aspire to follow in her footsteps, and I feel I'm getting closer to that dream—thank you, Michelle.

I am also deeply grateful to Zack for being an exceptional advisor. Over the past five years, he has not only taught me but also become family. His generosity and patience have been instrumental in shaping me into the scientist I am today.

I would like to thank my committee members for their valuable input throughout the years. Each meeting left me feeling more confident in my progress, and I appreciate their support.

Lastly, a heartfelt thanks to my friends. Though I can't name everyone, they know how much I value their presence in my life. I want to give a special shoutout to Quanita, my partner throughout this PhD journey; I couldn't have made it without her.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	v
CHAPTER	
1 INTRODUCTION AND LITERATURE REVIEW	1
Purpose of this study	1
Introduction to Polycomb Repressive Complexes	1
Control of H3K27me3 and PRC2 in <i>Neurospora crassa</i>	9
An experimental system to uncouple H3K27me3 assembly from maintenance ...	13
References	17
Figures	46
2 HISTONE DEACETYLASE-1 IS REQUIRED FOR EPIGENOMIC STABILITY IN <i>NEUROSPORA CRASSA</i>	49
Abstract	50
Introduction	51
Materials and Methods	55
Results	58
Discussion	65
References	70
Figures	99
Tables	108

3	POSITIVE FEEDBACK BETWEEN H3K27ME3, ISW, AND H3K36ME3 PROMOTES MITOTIC INHERITANCE OF H3K27ME3.....	109
	Abstract.....	110
	Introduction.....	110
	Materials and Methods.....	114
	Results	117
	Discussion.....	123
	References.....	125
	Figures.....	155
	Tables.....	164
4	DISCUSSION	166
	Histone Deacetylase 1 (HDA-1) regulates PRC2	167
	Uncoupling H3K27me3 establishment and maintenance in <i>Neurospora</i>	168
	Boundaries regulating H3K27me3 spreading in <i>Neurospora</i>	170
	References.....	173
APPENDICES		
	A) ECTOPIC TARGETING OF H3K27ME3 IS SUFFICIENT FOR GENE REGULATION.....	202

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Purpose of this study

H3K27 methylation, a post-translational histone modification, functions as a regulatory mechanism that modulates the activity of developmental genes in diverse organisms such as plants, animals, and fungi. This essential process enables the precise control of gene expression during various stages of development. In this dissertation, the model organism *Neurospora crassa* was utilized to gain insights into the complex processes associated with establishing, maintaining, and spreading of H3K27 methylation. I showed that *Neurospora*'s Histone Deacetylase 1 (HDA-1) regulates H3K27me3 and PRC2 to maintain genome stability. I also developed a novel experimental system that enabled us to explore how silent heterochromatic H3K27me3 domains are assembled and maintained. The work presented in this dissertation has increased our understanding of how H3K27me3 domains in *Neurospora* are regulated.

Introduction to Polycomb Repressive Complexes

Polycomb group (PcG) proteins were first characterized in *Drosophila melanogaster* for their role in repressing homeotic (Hox) genes, which are essential for segmentation (Kassis et al., 2017a; E. B. Lewis, 1978). PcG proteins assemble into two large complexes that modify histones post-translationally. The first of these complexes is Polycomb Repressive Complex 1 (PRC1), which is responsible for the ubiquitination of lysine 118 on histone H2A (H2AK118ub) in *Drosophila*. The second is Polycomb Repressive Complex 2 (PRC2), which is responsible for the methylation (di- and tri-) of lysine 27 on histone H3 (H3K27me2/3) (Schoeftner et al., 2006).

PRC1 and PRC2 operate within a positive feedback loop where PRC2 activity is enhanced by H2Aub, while PRC1 contains a binding protein that recognizes H3K27me3. PcG proteins are conserved in animals and plants, but fungi only have PRC2 (Guitton & Berger, 2005). These proteins are notably absent from preeminent eukaryotic model systems such as *Saccharomyces cerevisiae*. Since fungi do not have PRC1, this review will focus on characterizing the role of PRC2 among eukaryotes. The four essential PRC2 subunits that make up PRC2 in *Drosophila* are enhancer of zeste (E(z)), suppressor of zeste 12 (Su(z)12), extra sex combs (Esc), and nucleosome remodeling factor (Nurf55, also called p55). These core subunits of PRC2 have also been identified in mammals, fungi, plants, red algae, and diatoms (Vijayanathan et al., 2022). Compared to *Drosophila*, mammals and plants have multiple versions of the core subunits. The core PRC2 subunits in mammals are made up of two methyltransferases (EZH1 and EZH2), one copy of SUZ12 and EED, and either RBBP4 or RBBP7 (Fischer et al., 2022; Sharaf et al., 2022). The model plant system *Arabidopsis thaliana* contains 12 homologs of the *Drosophila* PRC2 subunits. The H3K27me3 methyltransferase *EZ* is encoded by three homologs (*CURLY LEAF*, *MEDEA* and *SWINGER*; *CLF*, *MEA* and *SWN*) (Hennig & Derkacheva, 2009a; Ruta et al., 2019). PRC2 plays different roles in plants, animals, and fungi, and those roles will be explored below.

Polycomb Repressive Complex in higher eukaryotes

PRC2 is vital for silencing a subset of developmental genes in plants and animals. The loss of core PRC2 subunits results in a drastic reduction of H3K27me3 levels in PRC2 target genes, which leads to severe developmental defects in plants and animals (Baile et al., 2022; Cyrus et al., 2019; Faust et al., 1995; A. He et al., 2012; Huh et al., 2007; M. Luo et al., 1999; Mozgova et al., 2015; Struhl, 1981). The processes regulated by PRC2 include stem cell

differentiation, embryonic development, tissue homeostasis, and multicellular development. H3K27me3 deposited by PRC2 marks a large subset of genes in plants. About 20-25% of the genes in the model plant system *Arabidopsis* are marked by H3K27me3, and most of these genes are lowly expressed (Guitton & Berger, 2005, 2005; Lafos et al., 2011; Makarevich et al., 2006; Mathieu et al., 2005; X. Zhang et al., 2007a). One critical process that is regulated by PRC2 in plants is vernalization, which allows plants to sense their environment and flower only after winter (Guitton & Berger, 2005). This process is controlled by the floral repressor gene known as *FLOWERING LOCUS C (FLC)*. This gene is silenced during vernalization by PRC2, which prevents the flower from flowering during the winter (Bastow et al., 2004; Dennis et al., 2006; Gibbs et al., 2018; J. Lee et al., 2015; Sheldon et al., 2009; Sung & Amasino, 2004). PRC2 has also been shown to regulate other essential processes such as stress response, cellular response, and plant metabolism (Godwin & Farrona, 2022). The role of PRC2 in plants extends far beyond its functions in regulating the *Arabidopsis* genome. PRC2 regulates developmental genes in important agricultural plants such as maize and rice, which are major agricultural products that serve huge societal roles (G. He et al., 2010; Makarevitch et al., 2013). Thus, it is crucial for us to understand how PRC2 regulates the development of these plants.

The dysregulation of Polycomb proteins has been associated with a range of human developmental diseases. One example of such a disease is Weaver Syndrome. Patients affected with Weaver Syndrome have a heterozygous mutation in either the *EZH2* or *EED* genes, which encode PRC2 subunits (Gibson et al., 2012; Imagawa et al., 2017). These patients exhibit symptoms such as tall stature with or without a large head size (macrocephaly), an overgrowth of various tissues, intellectual disability, and high cancer susceptibility (Weaver et al., 1974). The reduction of PRC2 has also been linked to other diseases, such as diabetes. The PRC2 subunits

EZH2 and EED were shown to regulate pancreatic β cells, suggesting that PRC2 might regulate genes associated with diabetes pathogenesis (Chen et al., 2009). Researchers have also linked PRC2 to neurodegenerative diseases. A study in mice showed that when PRC2 was depleted in striatal neurons, there was an upregulation of PRC2-targeted genes that are important for protecting neurons against degeneration (von Schimmelmann et al., 2016). These examples show that PRC2 plays many roles and might be involved in other diseases that have not been explored yet.

The role of PRC2 in cancer has been widely explored. Multiple human cancers have been linked to a loss of function of core PRC2 subunits. The overexpression of the catalytic subunit of PRC2, EZH2, has also been used as a marker for many cancers. EZH2 is shown to be overexpressed in cancers such as melanoma, colorectal, endometrial, lymphoma, breast, and prostate cancer (Bracken et al., 2003). Loss-of-function and gain-of-function mutations have been reported in humans, indicating that PRC2 plays a role in tumor suppression in some cancers and acts as an oncogene in others (Laugesen et al., 2016; Varambally et al., 2002). A common cause of pediatric glioblastomas is a loss-of-function mutation where lysine 27 in histone H3 is replaced with methionine (K27M). These H3 K27M mutations occur in about 80% of diffuse midline gliomas (DMGs) (Karremann et al., 2018; Kurdi et al., 2022). It was reported that this mutation inhibits the catalytic activity of the PRC2 subunit, EZH2 (Bender et al., 2013; P. W. Lewis et al., 2013; Venneti et al., 2013). EZH2 has also been implicated in melanoma. A point mutation in EZH2 where tyrosine 641 (Y641) was replaced with phenylalanine (F) led to an upregulation of genes found in melanoma cells (Zimmerman et al., 2022). The role of PRC2 in eukaryotes is not limited to disease. In mice, EZH2 was shown to be required for the early development of intestine integrity and caudal fin regeneration in zebrafish (Dupret et al., 2017;

O'Carroll et al., 2001). PRC2 is also essential for multiple physiological processes, such as X chromosome inactivation. X chromosome inactivation is when one of the two X chromosomes becomes inactivated during development (Plath et al., 2003; Schoeftner et al., 2006). PRC1 and PRC2 have been implicated in silencing the genes on the inactive chromosome. Examples of X inactivation impacting traits in nature include the patterning of tortoise shells and the coat-color patterning of calico cats. Due to its importance in so many processes, it is critical that we understand the mechanisms that regulate this protein complex.

Polycomb Repressive Complex in fungi

The role of PRC2 has not been thoroughly investigated in simple multicellular organisms like fungi. Homologs of PRC2 subunits initially characterized in *Drosophila* have also been identified in the ascomycetes *Neurospora* and *Fusarium* and the basidiomycete *Cryptococcus* (Connolly et al., 2013; Dumesic et al., 2015; Jamieson et al., 2013a; Ridenour et al., 2020; Stuwe et al., 2015). PcG proteins are absent from yeast models *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* (Freitag, 2017). The first functional Polycomb system in fungi was identified in the filamentous ascomycete fungus *Neurospora crassa*. Both metazoan and fungal PRC2 core subunits are similar, and PRC2 is responsible for depositing all the H3K27me3 found in the genome (Jamieson et al., 2013a). Deletion of *kmt6/EZH2* in *Fusarium graminearum* and *Ezh2* in *C. neoformans* resulted in a complete loss of H3K27me3 (Connolly et al., 2013; Dumesic et al., 2015). H3K27me3 deposited by PRC2 marks a significant number of genes in fungal genomes: 7% of genes in the *Neurospora* genome and approximately 30% in the *F. graminearum* genome. These genes marked by H3K27me3 have been reported to play pivotal roles in the growth and development of multiple fungi.

In the fungal species *Fusarium fujikuroi* (Studt et al., 2016) and *Fusarium graminearum* (Connolly et al., 2013), loss of H3K27me3 caused the upregulation of genes involved in production of secondary metabolites. Secondary metabolites are compounds produced by fungi for survival benefits. These products are not essential for fungal growth, but they help the fungus survive in its environment. Penicillin is an example of a secondary metabolite produced by the fungus *Penicillium* (Avalos & Limón, 2022; Keller et al., 2005). Some, but not all, fungal secondary metabolites are beneficial and can be used for medical purposes. Many fungal secondary metabolites, such as mycotoxins produced by the pathogen *Fusarium graminearum*, are toxic. *Fusarium graminearum* infects wheat, barley, and other cereal crops worldwide (Kelly & Ward, 2018) and causes a disease known as *Fusarium* head blight. This disease leads to the destruction of grains and the production of toxic metabolites such as deoxynivalenol. Humans and animals that eat the affected crops suffer from immune, gastrointestinal, and reproductive failure. This also affects crop yield, leading to billions of dollars being lost. Therefore, understanding how PRC2 silences these clusters is essential. This knowledge can be used to reduce the effects of mycotoxins and generate new secondary metabolites that are beneficial to humans and animals.

PRC2 is also important for pathogenicity. It has been reported that the core PRC2 subunits Kmt6-Suz12-Eed are required for fungal pathogenicity in *Magnaporthe oryzae*, which causes the disease rice blast (Wu et al., 2022a). Rice is one of the most consumed foods around the world, and rice blast poses a massive threat to the human population by affecting millions of rice crops globally. *Kmt6* is the H3K27me3 methyltransferase in *M. oryzae*, and loss of *kmt6* leads to reduced sporulation and a decrease in the pathogenic effect of the fungus in rice and barley (Pham et al., 2015). In *Ustilaginoidea virens*, the causal agent of the rice false smut disease, loss

of H3K27me3 led to reduced growth, conidiation, and pathogenicity in *U. virens* (Meng et al., 2021). PRC2 also regulates fungal effector genes, which code for proteins that help promote fungal colonization with a host (Constantin et al., 2021). *F. graminearum* is unable to infect tomatoes in the absence of PRC2 (Connolly et al., 2013). PRC2 has also been shown to control the symbiotic relationship between the fungus and its host. Both H3K9me3 and H3K27me3 were shown to regulate the expression of symbiosis-specific genes, which enable *Epichloë festucae* to form symbiotic relationships with its perennial ryegrass host (Chujo & Scott, 2014b). All these examples highlight the important role that PRC2 plays in the fungal kingdom.

Polycomb Repressive Complex in Neurospora crassa

The discovery and characterization of PcG complexes in fungi have provided a simple model for further exploration of the Polycomb system. The first fungal PRC2 complex was characterized in *Neurospora crassa*. Homologs of the human core PRC2 components EED (EED), SUZ12 (SUZ12), and EZH2 (SET-7), as well as RBBP4/7 (NPF) were identified in *Neurospora* (Jamieson et al., 2013a) (Figure 1.1). PRC2 is responsible for depositing all the H3K27me3 found in *Neurospora*, and H3K27me3 makes up 7% of the *Neurospora* genome. Deletion of the *set-7* (*Ezh/KMT6* homologs) gene does not affect the growth of *Neurospora* (Jamieson et al., 2013a). However, recent unpublished work from our lab suggests that in the absence of H3K27me3, there is an upregulation of genes involved in sexual development, specifically during the perithecial stage. H3K27me3-deficient mutants develop aberrant perithecia structures (Wiles et al., 2020). *Neurospora* also possesses other epigenetic machineries such as RNAi, DNA methylation, and H3K9me3 (Aramayo & Selker, 2013).

Constitutive heterochromatin in Neurospora crassa

The *Neurospora* genome is composed of euchromatin and heterochromatin. Euchromatin is an open form of chromatin that is lightly packaged and easily accessible to transcription factors. Euchromatin is enriched in acetylated histones H3 and H4 and H3K4 methylation (H3K4me), while genes marked by heterochromatin are densely compacted, gene-poor, and transcriptionally inactive (Tamaru, 2010). Euchromatin and heterochromatin have different levels of accessibility to transcriptional factors and DNA-binding proteins. In *Neurospora*, promoters marked with histone acetylation correlated with accessible chromatin, while those marked with heterochromatic features were linked to inaccessible chromatin (Ferraro et al., 2021).

Heterochromatin is further divided into two subtypes: constitutive and facultative heterochromatin. Constitutive heterochromatin is typically found in large blocks near centromeres and telomeres. Heterochromatin is also interspersed all along the chromosome arms. It primarily consists of repetitive DNA sequences (Dimitri et al., 2009). Constitutive heterochromatin is important for genome stability and cellular functions, such as chromosome organization and inheritance. Mice lacking H3K9me3 display genome instability and increased rates of developing cancers such as lymphomas (A. H. Peters et al., 2001). Constitutive heterochromatin has also been linked to DNA repair in *Drosophila* and *S. pombe* (Peng & Karpen, 2009).

Constitutive heterochromatin in *Neurospora crassa* is permanently silent and found at gene-poor, repetitive, AT-rich DNA sequences. The pathway in *Neurospora* is well defined, as shown in Figure 1.2. H3K9me3 is catalyzed by the histone methyltransferase DIM-5, which is part of the DCDC complex (DIM-7/DIM-9/DDB1/CUL4) (Tamaru et al., 2003). The H3K9me3 deposited by DIM-5 is bound by Heterochromatin Protein 1 (HP1) (Freitag et al., 2004). HP1 silences heterochromatin domains in *Drosophila*, fission yeast, and mammals (Bannister et al.,

2001; Hines et al., 2009; Seman et al., 2023). HP1 forms two complexes that interact with heterochromatin in *Neurospora*. The first is an HP1/DIM-2 complex. DIM-2 is the DNA methyltransferase responsible for all the DNA methylation in *Neurospora* (Honda & Selker, 2008). The second HP1-bound complex is HCHC (HP1/CDP-2/HDA-1/CHAP), which silences heterochromatin by removing acetyl groups from histones. The formation of constitutive heterochromatin is also essential for maintaining genome stability in *Neurospora*. H3K9 methylation is required for sexual development and normal vegetative growth in *Neurospora*. Loss of DNA methylation and the H3K9me3 methyltransferase complex DCDC led to chromosome segregation defects and hypersensitivity to the DNA-damaging agent methylmethanesulfonate (MMS) (Z. A. Lewis et al., 2010a). Heterochromatin formation is crucial for *Neurospora crassa* and other organisms, but the mechanisms regulating PRC2 and heterochromatin remain poorly understood.

Control of H3K27me3 and PRC2 in *Neurospora crassa*

The regulation of PRC2 activity and H3K27me3 levels in *N. crassa* is influenced by several factors, including constitutive heterochromatin machinery, other histone modifications such as H3K36me3 and chromatin remodeling complexes such as IMITATION SWITCH (ISWI). I will expand on the roles of these factors in regulating *Neurospora* PRC2 below.

The antagonistic relationship between PRC2 and constitutive heterochromatin

H3K27me3 (facultative heterochromatin) and H3K9me3 (constitutive heterochromatin) mark different genome regions, but both marks have been shown to influence each other. Loss of mouse cells' H3K9me3 methyltransferase, SUV39H, leads to H3K27me3 redistribution (A. H. Peters et al., 2001). H3K27me3 redistribution was observed in severely DNA-hypomethylated mouse somatic cells (Reddington et al., 2013). Loss of DNA methylation in *Arabidopsis* and

mouse embryonic stem cells triggers the movement of H3K27me3 from facultative heterochromatin (Hagarman et al., 2013; Mathieu et al., 2005). These results revealed that DNA methylation and H3K9me3 are required for appropriate Polycomb-mediated gene repression by constraining PRC2 targeting. Fungi such as *Neurospora* also have DNA methylation and H3K9me3. Does H3K9me3 and DNA methylation also regulate PRC2 and H3K27me3 in *Neurospora*?

In *Neurospora crassa*, loss of the H3K9 MTase or HP1 triggers the global redistribution of H3K27me3 from normal PRC2-targeted domains to constitutive heterochromatin domains (Basenko et al., 2015; Jamieson et al., 2016) (Figure 1.3). The redistribution of H3K27me3 in *Neurospora* resulted in genotoxic stress and defective growth (Basenko et al., 2015). What about DNA methylation? Contrary to what was reported in mammals and *Arabidopsis*, the loss of DNA methylation (DCDC) had no impact on H3K27me3 in *Neurospora* (Jamieson et al., 2013a). These studies show that H3K27me3 and PRC2 are regulated differently in mammals, plants, and fungi. Since PRC2 targeting in *Neurospora* was solely regulated by H3K9me3 and not DNA methylation, we wanted to further explore the mechanisms that control the crosstalk between these two heterochromatic marks. We focused on the histone deacetylase complex HCHC (HP1/CDP-2/HDA-1/CHAP), which silences heterochromatin. We followed up on previous studies that reported the roles of HP1 in targeting PRC2 to the correct domains. We explored the roles of the other HCHC components (HDA-1, CDP-2, and CHAP) in silencing PRC2-targeted domains. This study also looked at the roles of other histone modifications in regulating PRC2.

H3K36 methylation regulates PRC2

In *Neurospora*, H3K36me₃ is catalyzed by two methyltransferases, ASH1 and SET-2. ASH-1 adds mono- and di-methyl groups to histone H3 at lysine 36 (H3K36) and occurs at transcriptionally silent genes. SET-2 is targeted by RNA polymerase II and catalyzes H3K36 trimethylation along gene bodies of actively transcribed genes (Adhvaryu et al., 2005). ASH1-catalyzed H3K36me regulates PRC2. In *Neurospora*, it was reported that ASH1-mediated H3K36 methylation acts upstream of PRC2, and H3K36me₃ recruits H3K27me_{2/3} to reinforce the repression of inactive genes (Bicocca et al., 2018). Bicocca et al. (2018) also proposed that ASH-1/H3K36me₃ modulates the accumulation of H3K27me_{2/3} both positively and negatively. In another filamentous fungus, *Magnaporthe oryzae*, it was recently reported that H3K27me₃/PRC2 recruits ASH-1/H3K36me₃ and vice versa (M. Xu, Sun, et al., 2024; M. Xu, Zhang, et al., 2024). The results in *Magnaporthe oryzae* contrast those reported in *Neurospora*.

The role of H3K36 methylation in regulating PRC2 in fungi also contrasts those reported in higher eukaryotes and yeast. In these systems, ASH1-mediated H3K36me counteracts Polycomb-associated silencing (Dorigi & Tamkun, 2013; Shirane et al., 2020). In *Drosophila*, ASH1 promotes the expression of HOX genes by counteracting repressive methylation of H3K27 by Polycomb group proteins (Dorafshan et al., 2017; Miyazaki et al., 2013). There is contradictory data in the literature/a gap in knowledge about the role of ASH1/H3K36me in regulating PRC2. My dissertation addresses some of these gaps/contradictory results currently present in the literature.

Other factors regulating PRC2 in Neurospora crassa

Compared to plants and animals, the mechanisms that regulate the establishment and maintenance of PRC2 occupancy in fungi remain poorly understood. In plants and animals, PRC2 recruitment and the silencing of heterochromatin domains is regulated by PRC1 and

PRC2. These Polycomb proteins work together to ensure the chromatin states at these domains are established and maintained. In contrast, fungi have PRC2 but lack PRC1. Since fungi lack PRC1, this suggests that there are other factors assisting PRC2 in ensuring that PRC2-targeted domains are silent through development. Several factors have been identified in *Neurospora* that are required for PRC2 regulation. The first is the histone variant H2A.Z. H2A.Z is required for normal H3K27me3 patterns in *Neurospora crassa* (Courtney et al., 2020a). In humans, PRC2 is associated with accessory proteins. A PRC2-Associated Subunit (PAS) was identified in *Neurospora* that is required for depositing subtelomeric H3K27me3 in the genome (McNaught et al., 2020). Finally, the chromatin remodeling factor ISWI was shown to regulate PRC2 activity (Deuring et al., 2000; Wiles et al., 2022a).

IMITATION SWITCH (ISWI) regulates PRC2 in Neurospora crassa

Nucleosome positioning modulates chromatin states and the activity of transcription machinery. The ATP-utilizing chromatin assembly and remodeling factor (ACF) complex is a key protein complex responsible for positioning nucleosomes on DNA (T. Ito et al., 1997). This complex was first characterized in *Drosophila melanogaster* and shown to be involved in regulating PRC2-targeted genes (Fyodorov et al., 2004; S. Ito et al., 2024; Scacchetti et al., 2018). The ACF complex is made up of the ATPase, Imitation Switch (ISWI), and the accessory subunit ACF1 (T. Ito et al., 1997). ACF contributes to gene repression by acting as a global nucleosome spacer (Deuring et al., 2000; Scacchetti et al., 2018).

In *Neurospora*, the *Drosophila* homolog of ISWI was shown to be required for the repression of PRC2-targeted genes (Kamei et al., 2021). The normal patterns of H3K27 and H3K36 methylation were affected in the absence of *Neurospora* ISW (Kamei et al., 2021), suggesting that both ASH1 and PRC2 function is disrupted in the absence of ISW. ISWI proteins use their

ATPase activity to create ordered arrays of nucleosomes in vitro and in vivo (T. Ito et al., 1997). Kamei et al. (2021) showed that, in *Neurospora*, ISW regulates PRC2 activity via its ATPase domain. A model was proposed where ISW is upstream of both PRC2 and ASH1. ISWI samples chromatin and uses its nucleosome remodeling activity to create transcriptionally repressed chromatin, which recruit ASH1 and PRC2 to stabilize the repression of PRC2-targeted domains. This model and data suggest that, in *Neurospora*, ISW plays a role in either the assembly or maintenance of repressed chromatin states. In this dissertation, I developed an experimental system that allowed us to decipher if ISW plays a role in the assembly or maintenance of silent heterochromatin domains.

An experimental system to uncouple H3K27me assembly from maintenance

DNA is wrapped around core histone octamers to form nucleosomes (Kornberg & Lorch, 1999). These histones are modified with various post-translational modifications, including methylation, acetylation, phosphorylation, and ubiquitination (J. M. Lee et al., 2023; Ramazi & Zahiri, 2021; Yang & Li, 2020). These modifications can alter chromatin structure. Some modifications make the chromatin more open and accessible, while others make it more compact and repressive. During cell division, these histones are replicated and distributed to daughter cells (Escobar et al., 2019; Reverón-Gómez et al., 2018a; Schlissel & Rine, 2019). The disrupted histone modification patterns are recognized and maintained by specific enzymes, ensuring that the same epigenetic state is inherited from the parental strands to the daughter strands. These chromatin modifications serve a critical role in epigenetic inheritance by enabling the transmission of gene expression patterns across generations. They can influence and regulate the expression of genes without altering the DNA sequence. They also regulate key biological and cellular functions and are involved in many disease states (Kleinjan & Lettice, 2008; Park et al.,

2022). Though these chromatin modifications are important for development, only some chromatin modifications are epigenetically inherited.

H3K9 and H3K27 methylation are associated with transcriptional repression and heterochromatin formation. These marks are stably inherited from one generation to the next. The maintenance and inheritance of these marks are important for various biological processes, including development and adaptation. Both H3K9 and H3K27me use a "read-write" mechanism to ensure the stability and regulation of gene expression across generations (Brickner, 2023). The "read-write" mechanism describes the relationship between various enzymes and protein complexes that can recognize specific epigenetic marks and subsequently modify chromatin to maintain or alter cellular states. During DNA replication, H3K27me₃ patterns are deposited by the PRC2 catalytic subunit EZH2 in mammals (Mozgova et al., 2015; Yang & Li, 2020). PRC2 can also recognize the existing H3K27me₃ on the parental strand and bind to it using its EED subunit. PRC2 also recruits PRC1, which contains protein domains that can bind H3K27me₃. The actions of PRC1 and PRC2 ensure that the repressive state is passed on to daughter cells (Angrand, 2022; Schoeftner et al., 2006, 2006). H3K9me₃ also has a similar "read-write" mechanism. H3K9me₃ is recognized by heterochromatin protein 1 (HP1) and binds H3K9me₃. Once bound, HP1 recruits additional factors, such as histone deacetylases, to help maintain the structure of heterochromatin and silence transposable elements and repetitive sequences (Hines et al., 2009; Jamieson et al., 2016; Peng & Karpen, 2009; Seman et al., 2023). The establishment and maintenance of these repressive chromatin modifications are two distinct processes.

The establishment of silent heterochromatin is defined as the initial formation and organization of densely packed chromatin that is marked with the hallmarks of heterochromatin, such as H3K9 or H3K27 methylation, while maintenance refers to the stable inheritance of the

established heterochromatic state during cell division and throughout the life of the cell (Blythe & Wieschaus, 2016; Ragunathan et al., 2015; Rusche et al., 2003; Seman et al., 2023). Separating the establishment, spreading, and inheritance of heterochromatin experimentally is difficult. First, these histone modifications are often involved in both processes. In PRC2, H3K27me3 is required for both the initial formation of heterochromatin and its stabilization during cell division. There are also positive feedback loops that exist between the modifications and the proteins that bind them. And what we know about one organism does not hold for another. One example is when we look at the role of PRC1 and PRC2 in fungi, higher eukaryotes, and plants. Fungi lack PRC1, so we cannot generalize findings regarding establishment and maintenance between organisms (Jamieson et al., 2013a). I built a system that allowed us to uncouple establishment and maintenance in *Neurospora crassa*. We used this system to determine how silent H3K27me3 domains are established and maintained. We also identified factors that regulate H3K27me3 establishment and maintenance at these PRC2-targeted domains.

In summary, my dissertation focused on addressing the gaps in knowledge associated with PRC2 and how PRC2 activity regulates H3K27me3 at PRC2-targeted domains. We know that PRC2 plays essential roles in development and other biological processes, but little is known about how these H3K27me3 domains are assembled or maintained over multiple generations. We also know that PRC2 does not act on its own. Rather, it interacts with other proteins to ensure that the heterochromatin is silent and stable. I explored the relationship between PRC2 and constitutive heterochromatin machinery, H3K36me3, and the chromatin remodeling complex, ISW. The results presented here expand on what we know about PRC2 regulation in the model fungus *Neurospora crassa*.

Dissertation Overview

The mechanisms regulating the establishment and maintenance of H3K27me3 in *Neurospora crassa* are not fully understood. In Chapter 2, we explored the crosstalk between the constitutive and facultative heterochromatin machinery. Specifically, the role of Histone Deacetylase 1 (HDA-1) in regulating H3K27me3 and PRC2. We saw that HDA-1 is important for genome stability in *Neurospora*. We reported global redistribution of H3K27me3 from PRC2-targeted domains to regions marked by constitutive heterochromatin. We also reported changes in the localization of other histone modifications, such as H3K36me3 and acetylation in the absence of *hda-1*. These findings underscore a novel function of histone deacetylases in the regulation of H3K27me3 and PRC2 in *Neurospora*.

In Chapter 3, we developed an experimental design to separate H3K27me3 establishment and maintenance. Using the tetR/O system, we saw that a tethered PRC2 subunit is sufficient to establish an ectopically silent H3K27me3 domain. The ectopic domain of H3K27me3 is sufficient to recruit H3K36 machinery. The artificial domain of H3K27me3 is maintained for multiple rounds of cell replication. Finally, we reported that a chromatin remodeling complex, ISW, is required for the maintenance of H3K27me3 over multiple rounds of cell replication. We also showed that ISW is required for the establishment of H3K36me3 at the artificial tetO array. Collectively, these results demonstrate that the establishment and maintenance of PRC2 and H3K27me3 in *Neurospora* are governed by distinct regulatory factors. The discussion in Chapter 4 presents a synthesis of these findings, contextualizes their significance, and explores potential future directions for research.

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Figures

Facultative heterochromatin

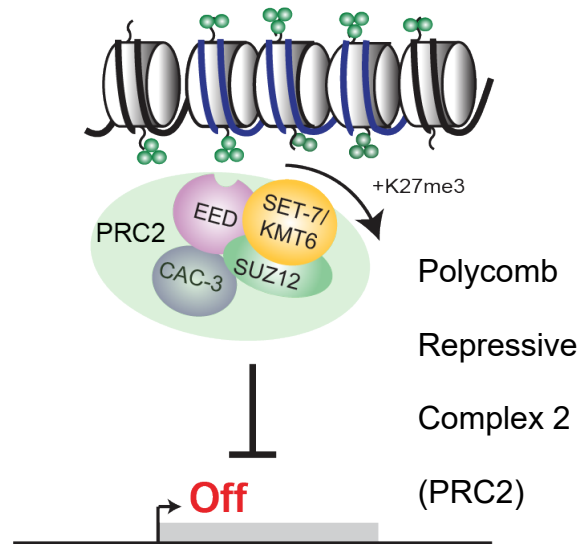


Figure 1.1: Facultative heterochromatin pathway in *Neurospora crassa*. Facultative heterochromatin in *Neurospora* is characterized by the deposition of H3K27me3 by Polycomb Repressive Complex 2 (PRC2). PRC2 catalyzes the deposition of H3K27me3 through the action of the histone methyltransferase SET-7, leading to gene repression.

Constitutive heterochromatin

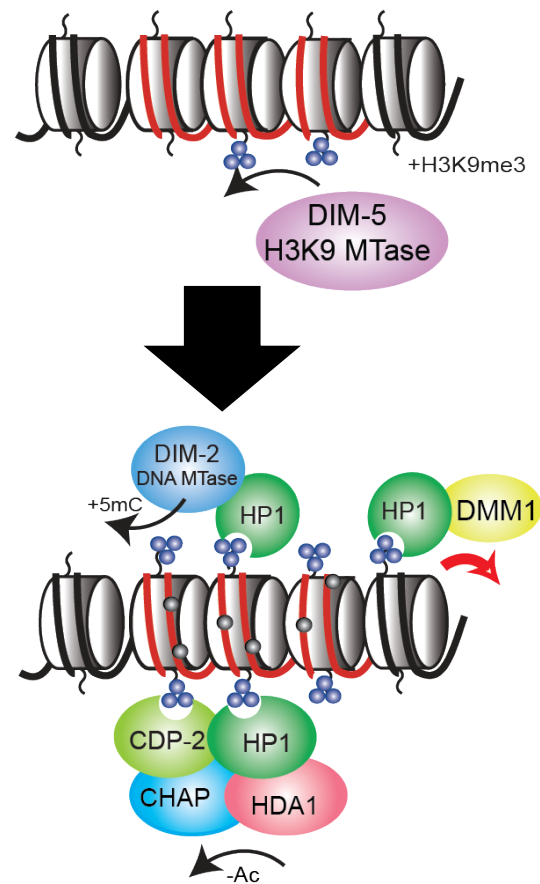


Figure 1.2: Constitutive heterochromatin pathway in *Neurospora crassa*. H3K9me3 is deposited by the methyltransferase DIM-5, marking regions of constitutive heterochromatin. Heterochromatin Protein 1 (HP1) is recruited and binds to these H3K9me3-marked regions. The HCHC complex, which includes histone deacetylase HDA-1, further enhances the silencing of the heterochromatin by removing acetyl groups from histones.

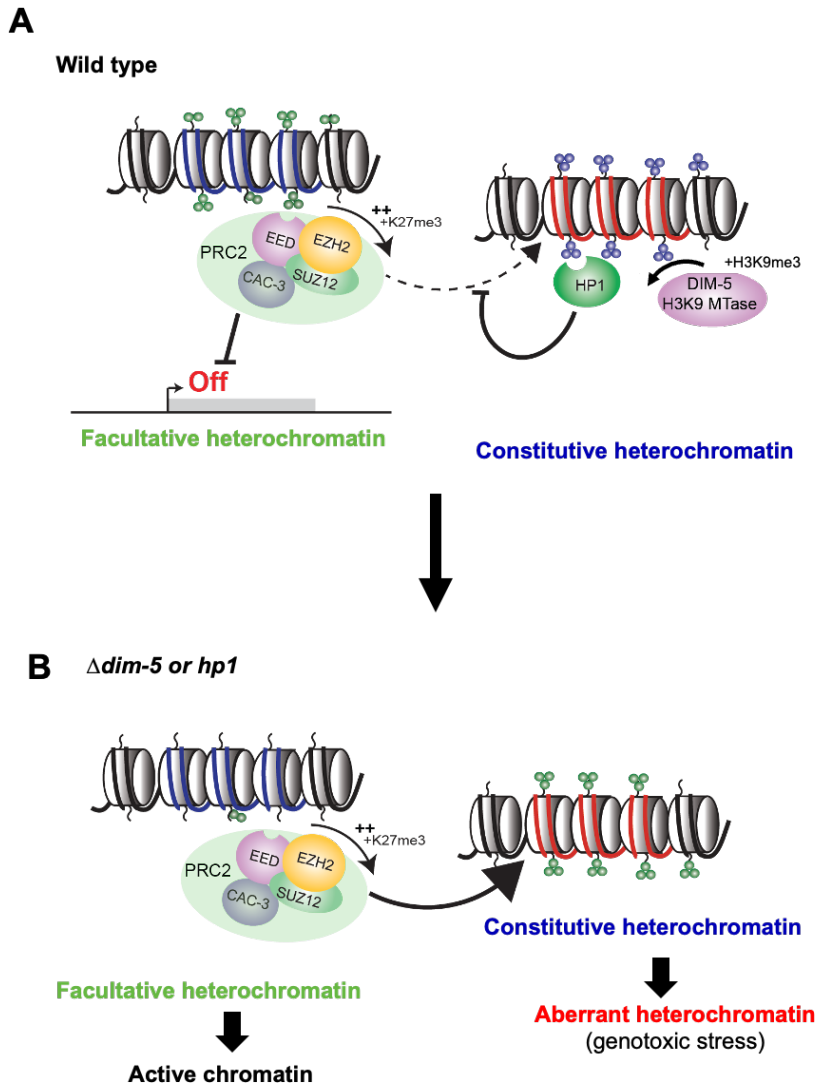


Figure 1.3: Model of Constitutive heterochromatin components preventing inappropriate PRC2 activity. (A) In wild type, HP1 activity prevents PRC2 recruitment to constitutive heterochromatin. (B) In the absence of the H3K9 methyltransferase DIM-5 or HP1, PRC2 invades constitutive heterochromatin, leading to aberrant redistribution of H3K27me3.

CHAPTER 2

HISTONE DEACETYLASE-1 IS REQUIRED FOR EPIGENOMIC STABILITY IN

NEUROSPORA CRASSA

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Abstract

Polycomb group proteins make up a family of multiprotein complexes essential for regulating stem cell identity, differentiation, and disease states. H3K27me₃ is catalyzed by a conserved Polycomb Repressive Complex 2 (PRC2). In *Neurospora crassa*, the pattern of H3K27me₃ depends on other chromatin regulators including the H3K36 methyltransferase ASH1, the chromatin remodeling enzyme IMITATION SWITCH, and components of constitutive heterochromatin including the H3K9 methyltransferase DEFECTIVE IN METHYLATION-5 and the H3K9me₃-binding protein HETEROCHROMATIN PROTEIN 1 (HP1). How constitutive heterochromatin impacts PRC2 activity is unclear. HP1 forms multiple protein complexes including the HCHC complex, which contains Histone Deacetylase 1 (HDA-1), and functions to remove acetyl groups from histones in constitutive heterochromatin. We carried out a genetic screen to identify genes required for Polycomb repression in *N. crassa*. We found that HDA-1 is required for repression of PRC2-targeted genes. In the absence of HDA-1, H3K27me_{2/3} is lost from typical PRC2-targeted domains and accumulates aberrantly at constitutive heterochromatin domains typically enriched for H3K9me₃. We also showed that Chromodomain protein 2 (CDP-2), another HCHC-deficient mutant, is important for proper localization of the H3K27methylation. Redistribution to constitutive heterochromatin domains was variable and incomplete in these two mutant strains, and mutant progeny obtained following a sexual cross displayed wild-type H3K27me₃ patterns. This raised the possibility that loss of HDA-1 activity leads to progressive changes in H3K27me₃ patterns over many nuclear division cycles. Consistent with this model, a newly constructed $\Delta hda-1$ strain displayed wild-type H3K27me₃ patterns that decayed as the strain was sequentially passaged. Together, our results show that H3K9me₃ recruits components of the HCHC deacetylation complex to prevent aberrant recruitment of PRC2 to constitutive

heterochromatin domains.

Introduction

Polycomb Group (PcG) proteins were first discovered in *Drosophila melanogaster*, where they played a role as epigenetic regulators of the homeotic (Hox) gene clusters (Duncan, 1982; E. B. Lewis, 1978; Struhl, 1981). These proteins form a variety of repressive chromatin modification complexes, including PRC1-type and PRC2-type complexes (Kassis et al., 2017b; Parreno et al., 2022). PRC2 is responsible for methylating histone H3 at lysine 27 (H3K27me1, 2, and 3), a key mark for transcriptional repression in animals, plants, and most fungi (Cao et al., 2002; Czermin et al., 2002a; Hennig & Derkacheva, 2009a; Jamieson et al., 2013a; Jiao & Liu, 2015; Kuzmichev et al., 2002; Schuettengruber et al., 2017). In plants, PRC2 is essential for controlling developmental processes and responses to environmental stimuli by modulating chromatin structure and gene silencing (Bastow et al., 2004; Makarevich et al., 2006; X. Zhang et al., 2007b). In animals, PRC2 maintains stem cell pluripotency, regulates cell differentiation, and is essential for epigenetic processes such as X-chromosome inactivation (Guitton & Berger, 2005; Plath et al., 2003; Vijayanathan et al., 2022; J.-R. Yu et al., 2019). In humans, improper regulation of H3K27me3 is linked to various diseases, including cancer and Weaver syndrome (Deevy & Bracken, 2019; Gibson et al., 2012; Imagawa et al., 2017; Laugesen et al., 2016; Varambally et al., 2002; Weaver et al., 1974). In fungi, PRC2 plays a crucial role in regulating gene expression associated with secondary metabolism and virulence, both of which are essential for fungal survival and pathogenicity (Connolly et al., 2013; Jamieson et al., 2013a; Keller et al., 2005; Wu et al., 2022a).

Due to the crucial biological roles of PRC2 and H3K27 methylation, there is a strong interest in understanding PRC2's regulatory mechanisms and function. This has mostly been

extensively studied in mammals, revealing complex mechanisms of PcG control (Bauer et al., 2016; Blackledge & Klose, 2021; Margueron & Reinberg, 2011a; Merini & Calonje, 2015b; Mozgova et al., 2015; Simon & Kingston, 2009; Uckelmann & Davidovich, 2021a; J.-R. Yu et al., 2019). PRC1 and PRC2 are recruited to target domains by DNA-binding proteins or each other (Brown et al., 2024; Schuettengruber et al., 2017; Shi et al., 2024; Tamburri et al., 2020). Besides recruitment, the activity of these complexes is regulated by several chromatin features, including chromatin density (Yuan et al., 2012), activating modifications (Barbour et al., 2020; Kalb et al., 2014a; Ohtomo et al., 2023), inhibitory modifications (Cookis et al., 2024; Goto et al., 2024; Li et al., 2017; C. Luo et al., 2018; Wei & Wu, 2022; M. Xu, Sun, et al., 2024), and RNA (Alecki et al., 2020; Balas et al., 2021; Cifuentes-Rojas et al., 2014; Kaneko et al., 2014; Klattenhoff et al., 2013; Song et al., 2023; Uckelmann & Davidovich, 2021b).

The core PRC2 components are conserved and were recently identified in multiple fungi, including *Neurospora* (Dumesic et al., 2015; Erlendson et al., 2017; Jamieson et al., 2013a; Jiao & Liu, 2015, 2015). Like mammals, PRC2 is responsible for all H3K27 methylation found in *Neurospora* and other fungi (Chujo & Scott, 2014b; Connolly et al., 2013; Dumesic et al., 2015; Jamieson et al., 2013a, 2016). Very little is known about how fungal PRC2 complexes are targeted to chromatin. Similar to mammals, PRC2 activity in *Neurospora* is influenced by multiple factors, such as histone variants (H2A-Z) (Courtney et al., 2020a), accessory proteins (CAC-3/NPF, EPR-1, and PAS) (Jamieson et al., 2013a; McNaught et al., 2020; Wiles et al., 2020), chromatin remodelers (ISWI and ACF) (Kamei et al., 2021; Wiles et al., 2022b) and other histone modifications (such as H3K36me3) (Bicocca et al., 2018). In mammalian systems, H3K36 methylation is known to inhibit PRC2 activity in vitro (Bartke et al., 2010; Gaydos et al., 2012; Musselman et al., 2012; Schmitges et al., 2011; Yuan et al., 2011). However, this inhibitory effect

has not been reported in fungi (Connolly et al., 2013). In *Neurospora*, H3K36 methylation is catalyzed by two enzymes, SET-2 and ASH-1. Deletion of SET-2 did not impact global patterns of H3K27me3 (Bicocca et al., 2018). In contrast, ASH-1 has been shown to positively and negatively regulate H3K27me3 levels. The H3K36 methylation catalyzed by ASH-1 overlaps with H3K27me3 domains, and the loss of ASH-1-dependent H3K36 methylation is linked to both reductions and increases in H3K27me2/3 across the *Neurospora* genome (Bicocca et al., 2018). These findings suggest a collaborative relationship between PRC2 and various histone modifications that collectively influence its regulatory functions.

Neurospora crassa also possesses other epigenetic machinery, such as DNA methylation and H3K9me3, which make up the constitutive heterochromatin pathway (Aramayo & Selker, 2013; Jamieson et al., 2013a, 2016). Constitutive heterochromatin in *Neurospora crassa* is marked by its AT-rich DNA (Z. A. Lewis et al., 2009). The presence of DNA methylation, hypoacetylation, and H3K9me3 characterizes this type of heterochromatin in *Neurospora*. The constitutive heterochromatin pathway observed in *Neurospora crassa* shares notable similarities with the mechanisms found in the fission yeast *Schizosaccharomyces pombe*, highlighting conserved strategies for gene regulation and genomic stability across diverse eukaryotic organisms (Al-Sady et al., 2013; Y. Zhang et al., 2008). The predominant marker of constitutive heterochromatin in *Neurospora* is H3K9 methylation. All H3K9me methylation in *Neurospora* is catalyzed by DIM-5, which is part of a complex known as the DIM-5/DIM-7/DIM-9/DDB1/CUL4 complex (DCDC) (Z. A. Lewis et al., 2010a; Tamaru et al., 2003). Deposition of H3K9me3 leads to the recruitment of Heterochromatin Protein 1 (HP1), which binds H3K9me3 and serves as a recruiting platform for other complexes (Freitag et al., 2004; Jamieson et al., 2016). One complex that binds to HP1 is the DNA methyltransferase DIM-2 (Honda & Selker, 2008; Kouzminova & Selker, 2001). DIM-

2 is responsible for all known cytosine methylation in *Neurospora*. HP1 also recruits the histone deacetylase complex known as HCHC (HP1/CDP-2/HDA-1/CHAP) (Gessaman & Selker, 2017; Honda et al., 2012; Scadden et al., 2023). DIM-2 and HCHC work together to properly establish and maintain constitutive heterochromatin. HCHC is required for proper regulation of DNA methylation in *Neurospora* (Honda et al., 2016).

PRC2 activity in fungi is also influenced by constitutive heterochromatin machinery. In *Neurospora*, the disruption of H3K9 methyltransferase and HP1 machinery results in a widespread loss of H3K27me3 from its typical domains, along with the emergence of aberrant H3K27me3 in regions characterized by constitutive heterochromatin (Basenko et al., 2015; Jamieson et al., 2016). *Neurospora* strains that lacked H3K9me3 or HP1 exhibited growth defects and were vulnerable to genotoxic stress (Sasaki et al., 2014; Tamaru & Selker, 2001). Loss of DNA methylation in *Neurospora* had no impact on PRC2 and H3K27me3 enrichment (Basenko et al., 2015; Jamieson et al., 2016). In mammals, depletion of DNA methylation or H3K9me3 induces significant global changes in the distribution of H3K27me3 in mammalian cells (Dumesic et al., 2015; Hagarman et al., 2013; Jin et al., 2011; Lindroth et al., 2008; Mathieu et al., 2005; Möller et al., 2019; A. H. F. M. Peters et al., 2003; Rose & Klose, 2014; Studt-Reinhold et al., 2024; Tariq & Paszkowski, 2004). This is also true for plant species such as *Arabidopsis*, where the loss of DNA methylation leads to the redistribution of H3K27me3 to demethylated transposons (Deleris et al., 2012). The interplay between PRC2 and heterochromatin in fungi, plants, and animals underscores the complexity of epigenome stability. We used the simple model fungus *Neurospora crassa* to explore the complexity between PRC2 and constitutive heterochromatin - specifically the role of Histone Deacetylase 1 (HDA-1) in regulating PRC2 activity.

The components of the HCHC complex are not essential in *Neurospora*. Here, we report

that strains deficient in HDA-1 exhibit significant alterations in the global distribution of H3K27me3, a key histone modification associated with transcriptional repression. These patterns of H3K27me3 redistribution in the HDA-1-deficient strain are reminiscent of the alterations observed in the absence of HP1, further emphasizing the interconnected nature of these epigenetic regulators. Moreover, our investigations reveal that the loss of HDA-1 leads to gradual and progressive genomic instability that manifests following multiple rounds of nuclear division. This instability underscores the importance of HDA-1 in maintaining genomic integrity. Collectively, these findings contribute to a deeper understanding of the mechanisms underlying epigenome stability in *Neurospora crassa* and highlight the pivotal role of HDA-1 in these processes.

Materials and Methods

Strains and growth conditions

Strains are listed in Table 2.1. Some of the strains were acquired from the *Neurospora* whole-genome knockout collection (Colot et al., 2006). We also created a new knockout strain of *hda-1*. We also used an *hda-1* his-3 strain that we complemented. Strains were grown in Vogel's minimal medium with 1.5% sucrose (Davis & de Serres, 1970).

Cleavage Under Targets & Release Using Nuclease (CUT&RUN)

CUT&RUN was performed based on a protocol adapted from the Henikoff lab (Skene & Henikoff, 2017). For *Neurospora* nuclei isolation, dH₂O was used to make a conidial suspension of the desired strain. 100 µL of the conidial suspension was added to 5 mL of liquid Vogel's medium and incubated overnight at 32°C shaken at 180 RPM. The mycelium was harvested the next day and placed on a petri dish on ice. Using a razor blade, the mycelium was chopped for a total of 2 minutes with 1 mL of nuclei isolation buffer (1M HEPES pH 7.5, 10 mM Spermine tetrahydrochloride, 1M KCl, 4M NaCl, 1M CaCl, 14.3M 2-Mercaptoethanol, 100% Triton X-100)

being added per 5 mL of cultured mycelium. Once the mycelium was completely fragmented in the nuclei isolation buffer, the solution was filtered through a miracloth into a 1.5 mL Eppendorf tube. The tube was centrifuged for 5 minutes at a speed of 1000 xg. The supernatant containing the nuclei was saved and analyzed as described in the following paper (Meers et al., 2019).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described in (Ferraro & Lewis, 2018) or H3K27me2/3 (Active Motif Mouse Monoclonal, Catalog # 39535, Lot# 16714012), H3K9me3 (Active Motif Rabbit Catalog # 39162), H3K36me3 (Catalog # ab9050) and Acetylated-Lysine (Catalog # 9441S). Sequencing libraries were created and analyzed as described in the following paper (Ferraro & Lewis, 2018). Libraries were sequenced by the Georgia Genomics Facility at the University of Georgia.

Data analysis

ChIP-seq reads were cleaned and trimmed using TrimGalore! Trimmed reads were mapped with BWA using the mem option with flagged secondary hits (bwa mem -M). Peaks were called using MACS2 using the broad option without input and with a q-value of 0.01. Bigwig files for visualization were made using deepTools bamCoverage with the following options: -bs 10 – normalize using CPM, --ignore Duplicates. Heatmaps were created using deepTools computeMatrix plotHeatmap. Browser tracks were created with IGV. The csaw package was used to quantify differential enrichment of H3K27me3, H3K9me3, H3K36me3, or Kac in wild type and *Δhda-1* strains (Lun & Smyth, 2016).

RNA-seq

RNA-seq reads were acquired from JGI Community Sequencing Grant 54039. Paired RNA-seq reads were first de-interleaved using bbmap's reformat.sh. Paired read files were then

trimmed using trimGalore!. Trimmed reads were mapped to the NC12 genome using HISAT2 with standard options. Read counts were created using featureCounts with the following options: -t exon -g gene_name -s2 -p --primary. A gtf of the assembled chromosome only was used. Counts to transcripts per million (TPM) normalization was performed in R. TPM read counts were used to make heatmaps with heatmap.2. Hierarchical clustering of standardized read counts was performed in heatmap.2 with the standard options (clustering_method = ward.D, clustering_distance_cols = Euclidian), and rows were scaled to give a z-score. Volcano plots were created in R with EnhancedVolcano.

Hi-C

In situ Hi-C was performed similarly to previous experiments, except that instead of generating spheroplasts, crosslinked nuclei were isolated. Briefly, 25mL cultures of 1x Vogel's and 1.5% sucrose were started from $\sim 1.0 \times 10^8$ conidia and grown overnight at 32°C with shaking at 200 rpm. Cultures were crosslinked with 1% [final] formaldehyde, quenched with 125mM Tris-HCl, pH 8.0, and the mycelial mat was isolated by vacuum filtration and placed in a petri dish on ice. Nuclear isolation buffer (5mL; 20mM HEPES pH 7.5, 80mM KCl, 20mM NaCl, 0.1mM CaCl₂, 15mM B-mercaptoethanol, 0.3% Triton, and 0.5mM Spermine tetrahydrochloride [freshly added]) was added and the mycelial mat was rapidly cut with a new razor blade. The diced mycelial mat/nuclear isolation buffer lysate was filtered through autoclaved Miracloth (Millipore Sigma, cat# 475855-1R) lining a funnel in a 15mL conical tube, and nuclei were collected by centrifugation at 4,500 rpm for 5 minutes. The pellet was washed with an additional 1mL nuclear isolation buffer, and nuclei pellets were stored at -80°C. Nuclei comprising at least 3.5 ug of DNA were then used for the remainder of the *in situ* Hi-C protocol, starting with 6.25% SDS treatment to make the nuclei porous, as previously described. *In situ*, Hi-C libraries were sequenced on a

NovaSeq 6000 as PE59 reads, and output fastq datasets were mapped to the *Neurospora crassa* reference genome, version 14 (nc14) (Rodriguez et al., 2022) with Bowtie2, and contact matrices were built using the “hicBuildMatrix” command of the hicExplorer software package. To compare to the previously published WT MseI Hi-C dataset, a number of total reads were extracted from the NKL9 MseI dataset that would give approximately equal numbers of valid reads for comparison to a WT N150 MseI Hi-C dataset (or reads were extracted from the WT MseI dataset when comparing to NKL10) using the sed command, and after mapping reads and building the contact matrix with the selected fastq files, the resulting contact matrices were compared using the hicCompareMatrices command of his explorer. Images were generated with the hicPlotMatrix command from hicExplorer and used for figure construction.

ChIP-seq was performed as before (Scadden et al., 2023), using antibodies specific to H3K9me3 (Active Motif, catalog # 39161) and H3K27me2/3 (Active Motif, catalog # 39535). ChIP-seq libraries were sequenced on a NovaSeq 6000 as SR118 reads and resulting fastq datasets were mapped to the nc14 genome using bowtie2. Output sam files were converted to bam files, sorted, and indexed with samtools, and made into bigwig files with Deeptools for display on IGV. IGV images were used for figure construction.

Results

HDA-1 is required for the repression of PRC2-target genes

To determine if one or more histone deacetylases are required for transcriptional repression of PRC2-target genes, we performed RNA-seq on 10 gene deletion strains lacking non-essential histone deacetylase enzymes. For each gene deletion strain, we examined the gene expression of 642 PRC2-target genes associated with H3K27me3 in the wild-type strain. These genes are silent in mycelia grown under standard laboratory conditions. The *histone deacetylase-1* ($\Delta hda-1$) strain

showed striking upregulation of the PRC2 target gene set, whereas other HDAC-deficient strains had modest or no impact on this gene set (Fig 2.1A). Replicate experiments for each gene deletion strain produced highly reproducible results. We confirmed this result by plotting a heatmap for all 642 PRC2-target genes, which revealed the upregulation of many genes within this group (Fig 2.1B). Differential expression analysis revealed that multiple genes were upregulated by more than 2-fold in the $\Delta hda-1$ mutant with a q-value of < 0.05 (Fig 2.1C).

HDA-1 exhibits genome-scale rearrangements in H3K27me3 patterns similar to $\Delta hp1$

To determine if HDA-1 is required for normal chromatin structure at PRC2-target regions, we performed CUT&RUN to examine the distribution of H3K27me3 across all seven *N. crassa* chromosomes. We observed significant losses of H3K27me3 from regions typically associated with this modification. These losses were accompanied by gains of H3K27me3 in constitutive heterochromatin regions typically marked by H3K9me3. The distribution of H3K9me3 and H3K27me3 across Linkage Group III is shown for wild type and $\Delta hda-1$ in Fig 2.2A-B. Replicate experiments produced similar results. Interestingly, gains in H3K27me3 were accompanied by apparent reductions in H3K9me3 (Fig 2.2A-B). To examine the global changes in repressive chromatin structure, we generated heatmaps to illustrate the level of H3K27me3 and H3K9me3 across wild-type PRC2-target domains (Fig 2.2C) and wild-type constitutive heterochromatin regions (Fig 2.2D). This confirmed widespread losses of H3K27me3 from typical PRC2-target domains and selected gains of H3K27me3 in some H3K9me3 regions. To confirm that epigenetic changes were caused by the loss of HDA-1, we introduced an *hda-1-3xflag* construct into the *his-3* locus of an $\Delta hda-1$ deletion strain and performed ChIP-seq. Wild-type patterns of H3K27me3 and H3K9me3 were restored in this complemented strain. This served as an important validation for our CUT&RUN procedures, which had not previously been developed for *N. crassa*. We next

asked if HDA-1 catalytic activity is required for normal H3K9me3 and H3K27me3 patterns. We introduced an *hda-1^{h225a}-3xflag* allele into the *his-3; Δhda-1* strain and performed ChIP-seq. The presumptive catalytically dead allele failed to rescue H3K9me3 and H3K27me3 patterning defects (Fig 2.2E-F). Importantly, both epitope-tagged proteins were expressed at similar levels (Fig 2.2G).

Δhda-1 mutants exhibit hyperacetylation at constitutive heterochromatin but not at PRC2-target genes

HDA-1-deficient cells exhibit hyperacetylation at constitutive heterochromatin domains (Honda et al., 2012, 2016; Scadden et al., 2023). We wanted to explore the relationship between lysine acetylation (H3Kac) and altered H3K27me3 in HCHC-deficient cells. We performed ChIP-seq to compare levels of acetylated lysine and H3K27me3 in the WT and *Δhda-1* strains. In WT, as reported previously, low acetylation levels were observed in both PRC2-repressed chromatin and constitutive heterochromatin domains marked by H3K9me3. In the *Δhda-1* strain, we observed hyperacetylation in H3K9me3-enriched heterochromatin domains but not in domains that were typically marked by H3K27me3 (Fig 2.3A). Interestingly, constitutive heterochromatin domains that show gains of H3K27me3 had lower levels of H3Kac compared to domains that did not gain H3K27me3. The inverse correlation between H3K27me3 and H3Kac was apparent when heatmaps of H3K9me3, H3K27me3, and H3Kac were generated across all WT H3K27me3 and WT H3K9me3 domains (Fig 2.3C).

In wild type, H3K27me3 co-occurs with ASH1-dependent H3K36 methylation, which is proposed to recruit the RPD3L histone deacetylase. We, therefore, asked if H3K36me2 or H3K36me3 displayed altered patterns in the HDA-1 mutant. We observed no enrichment of H3K36me2. This is presumably because SET-2 efficiently converts ASH1-dependent H3K36me2

to H3K36me3 genome-wide. In contrast, we observed marked gains of H3K36me3 in constitutive heterochromatin regions typically depleted for this modification (Fig 2.3B). H3K36me3 patterns overlapped those of H3K27me3, and this modification was inversely correlated with Kac levels (Fig 2.3C). Notably, the levels and patterns of Kac and K36me3 in gene regions were unaffected in the *hda-1* mutant, consistent with HDA-1's known role as a member of the heterochromatic HCHC complex (Supplementary Figure 2.1).

Δhda-1 strains have increased intra- and inter-heterochromatin interactions in the 3D genome

To assess if altered histone Post Translational Modifications (PTM) enrichment influences the three-dimensional (3D) organization of the *Neurospora* genome, we performed heterochromatin-specific chromosome conformation capture coupled with high-throughput sequencing in *Neurospora* nuclei (*in situ* Hi-C) (Rao et al., 2014; Rodriguez et al., 2022) The *Neurospora* genome forms a Rab1 chromosome conformation characterized by a centromere cluster independent of telomere bundles (Lieberman-Aiden et al., 2009; Rao et al., 2014; Torres et al., 2023), and the other ~200 AT-rich interspersed heterochromatic regions within the *Neurospora* chromosomes strongly associate over Megabases (Mb) of genomic distance. In Hi-C, the use of the restriction enzyme *MseI* (recognition sequence: T^ATAA) effectively captures heterochromatic contacts (Rodriguez et al., 2022). Previously, it was shown that intra- and inter-heterochromatic region interactions are strongly increased in a *Δcdp-2* strain due to an increase in histone acetylation (Scadden et al., 2023). To both confirm these previous results and assess if similar changes are observed in another HCHC mutant, we performed *MseI in situ* Hi-C in two independent *Δhda-1* strain isolates (NKL9, NKL10) from a backcross of *Δhda-1* to wild type; we also performed H3K9me3 and H3K27me2/3 ChIP-seq of both strains to assess strain-specific chromatin structure changes.

As previously shown, *MseI* Hi-C of a WT strain (Fig 2.4A) (Rodriguez et al., 2022) produces contact probability heatmaps at 20 kilobases (kb) resolution, in which each individual heterochromatic region shows strong intra-region interactions, highlighting the aggregation of chromatin within constitutive heterochromatic regions. Moreover, extensive long-range interactions between heterochromatic regions are readily observed (Fig 2.4A, 2.4E, for two individual chromosomes, Linkage Groups [LG] II and V). Similar contact probability heatmaps are observed for the $\Delta hda-1$ strains NKL9 and NKL10, except that strong inter-heterochromatic region interactions are more apparent (Fig 2.4B, 2.4F), although the inter-heterochromatic increases in the NKL10 heatmap are less apparent due to reduced numbers of valid reads in the NKL10 *MseI* dataset. Comparison of the WT contact probability heatmap to that of the $\Delta hda-1$ strains showed strong gains in interactions at the center of each heterochromatic region, as well as between heterochromatic regions, with the centromeric-interspersed heterochromatic region contacts being the most prominent (Fig 2.4C, 2.4G), consistent with previous results of a $\Delta cdp-2$ strain (Scadden et al., 2023). Our $\Delta hda-1$ *MseI* Hi-C data did show discrepancies in the changes of mid-range, off-diagonal contacts compared to those in a $\Delta cdp-2$ strain (Scadden et al., 2023), possibly reflecting strain-specific contacts or variability of individual Hi-C datasets.

To highlight these increases in contact probability and compare them to changes in heterochromatic histone PTM enrichment, we examined the WT strain, the $\Delta hda-1$ strain, and the changes in WT vs. $\Delta hda-1$ strain interactions between the centromere and the right chromosomal arm of LGs II and V in NKL9 and NKL10 strains; the NKL10 heatmaps requires a lower resolution to observe contact probability changes with reduced valid read numbers. We chose these two chromosomes because LG II in both NKL9 and NKL10 strains gains H3K27me_{2/3} in a $\Delta hda-1$ strain, while LG V does not gain H3K27me_{2/3} in NKL9 but does have substantial H3K27me_{2/3}

enrichment in NKL10 (Fig 2.4C, 2.4G); in fact, the centromeric enrichment of H3K27me_{2/3} appears to be inversely correlated to the amount of remaining H3K9me₃, with chromosomes depleted of H3K9me₃ having greater enrichment of H3K27me_{2/3}. Across LG II, the NKL9 and NKL10 $\Delta hda-1$ strains have increased contact probability between the LG II centromere and the distant interspersed constitutive heterochromatic regions at a similar level; the increased Hi-C read depth of the NKL9 dataset readily highlights the strong gains in inter-heterochromatic region contact probability. Similar gains are observed across all heterochromatic regions in these two chromosomes, independent of H3K27me_{2/3} enrichment changes in constitutive heterochromatic regions. These data suggest that loss of HCHC function causes contact probability promiscuity among constitutive heterochromatic regions within the heterochromatin bundle, possibly due to the hyperacetylation and increased H3K36me₃ enrichment upon loss of the HCHC histone deacetylase.

Other HCHC components are required for normal H3K27me₃

HDA-1 is a component of the HCHC complex (Heterochromatin Protein 1, Chromodomain Protein 2, HDA-1 and CDP-2/HDA-1-associated protein (CHAP) complex. HP1 is also required for normal H3K27me₃ patterns and exhibits a phenotype that is similar but not identical to the phenotype observed in $\Delta hda-1$. We next asked if other components of HCHC are required for normal H3K27me₃ localization. We performed ChIP-seq using an antibody for H3K27me_{2/3} in strains deficient for other members of the HCHC complex. Consistent with previous results, the heterochromatin protein 1 (Δhpo) strain exhibited widespread re-localization of H3K27me_{2/3} to constitutive heterochromatin regions (Fig 2.5A). We also observed a redistribution of H3K27me_{2/3} in $\Delta cdp-2$ from typical PRC2-target domains to constitutive heterochromatin domains, similar to $\Delta hda-1$. We did not observe significant changes in H3K27me₃ distribution in

the CHAP mutant. Although Δhpo , $\Delta hda-1$, and $\Delta cdp-2$ exhibited global changes in the pattern of H3K27me₃, inspection of the data revealed a distinct pattern of H3K27me_{2/3} enrichment in each strain (Fig 2.5B). We constructed heatmaps to visualize H3K27me₃ enrichment across all WT H3K9me₃ domains in each of the HCHC-deficient mutants. This confirmed that shared and strain-specific gains of H3K27me₃ occur in these strains, with the CHAP-deficient strain displaying no gains of H3K27me₃ in these regions (Figure 2.5C).

HDA-1 deficiency leads to progressive epigenome instability over hundreds of nuclear division cycles

Given the differences in H3K27me₃ patterns observed between individual $\Delta hda-1$ isolates and in individual HCHC-deficient strains, we hypothesized that H3K27me₃ may be a slow and stochastic process, taking place over many nuclear divisions. To examine the progression of epigenomic changes following deletion of *hda-1*, we generated a new $\Delta hda-1$ strain by split-marker transformation of the wild-type strain. Primary transformants were screened by PCR to identify a homokaryotic transformant. We then passaged the primary transformant three consecutive times by streaking out cells to isolate a single colony and then expanding that colony on a slant (referred here as $\Delta hda-1$ P1, P2, and P3; Fig 2.6A). Each passage corresponds to ~25 - 40 rounds of mitosis. Cells from each slant were then grown for an additional 18 hours in liquid culture medium and subjected to ChIP-seq for H3K27me₃, H3K36me₃, H3K9me₃, and Kac. We first visualized results for H3K27me₃ in the IGV genome browser (Fig 2.6B, 2.6C). The phenotype of the *hda-1* after passage 1 closely resembled the wild type with respect to H3K27me₃. We observed progressive loss of H3K27me₃ from native PRC2-target domains and gains of H3K27me₃ over the next two passages.

To compare the changes in H3K27me3 with alterations in other histone modifications, we used the csaw package to quantify enrichment of H3K27me3, H3K36me3, H3K9me3, and Kac in 300 base pair windows spanning all wild type H3K27me3-enriched domains and H3K9me3-enriched domains (Fig 2.6D). After passage 1, we observed subtle gains of H3K27me3 and H3K36me3 in some H3K9me3-enriched regions, but both modifications were retained at native PRC2-target regions. We observed little to no loss of H3K9me3, but the levels of Kac were increased relative to wild type across many of the H3K9me3-enriched regions. After the second passage, this redistribution was not instantaneous but occurred progressively over multiple generations, suggesting that the process is not random but rather follows a more ordered, cumulative trajectory. Furthermore, we noted that the redistribution of H3K27me3 was accompanied by broader epigenome instability. This instability was evident in several histone modifications, including global acetylation, H3K36me3, and H3K9me3. The accumulation of H3K27me3 ultimately results in the depletion of H3K9me3. These observations indicate that the loss of HDA-1 leads to a gradual, progressive redistribution of H3K27me3. This finding is significant as it highlights a complex interplay between various histone modifications. The epigenome instability observed in other modifications underscores the broader impact of HDA-1 deficiency on chromatin organization and stability, emphasizing the importance of our research in understanding these fundamental biological processes.

Discussion

In this study, we report that Histone Deacetylase-1 (HDA-1) is required for repression of PRC2-target genes. Of the ten gene deletion strains lacking non-essential histone deacetylase enzymes, only HDA-1 was required for the repression of many PRC2-targeted genes. HDA3, the component of RPD3, is not present in the knockout collection and, therefore, was not identified.

In the absence of HDA-1, H3K27me3 patterns underwent global redistribution like what was reported in the absence of Heterochromatin Protein 1 (HP1) (Jamieson et al., 2016). The redistribution of H3K27me3 in the *hda-1* mutant was also associated with slow and gradual epigenetic instability.

Loss of DNA methylation machinery leads to widespread H3K27me3 redistribution in mammals (Reddington et al., 2013). In *Neurospora*, the loss of HP1 and the H3K9 methyltransferase led to the depletion of H3K27me3 from PRC2-targeted domains and the gain of H3K27me3 at constitutive heterochromatin domains (Basenko et al., 2015; Jamieson et al., 2016). The disruption of DNA methylation had no impact on H3K27me3 in *Neurospora* (Jamieson et al., 2016). Jamieson et al. reported that the mutation of HCHC components (CHAP, HDA-1, and CDP-2) had no impact on H3K27 methylation patterns (Jamieson et al., 2016), in contrast to our results here. We showed that H3K27me3 moves to regions marked by constitutive heterochromatin in the absence of HDA-1 and CDP-2. The discrepancy between our results and those of the Selker lab can be explained by the slow progressive redistribution observed here. Jamieson et al. likely assayed H3K27me3 in HCHC-deficient strains that had recently been backcrossed.

We proposed a model where HDA-1 indirectly regulates the aberrant localization of H3K27me3 through progressive changes in HP1 localization or localization of HP1 condensates. HP1 undergoes liquid phase separation in vivo and facilitates the accumulation of enzymes, such as HDACs (Grewal, 2023; Keenen et al., 2021; Larson et al., 2017; Sanulli et al., 2019; Strom et al., 2017). We speculate that in *N. crassa*, HP1 condensates actively exclude PRC2 from constitutive heterochromatin. HDACs repress histone turnover and help to maintain a high density of H3K9me3 (Chory et al., 2019; Deal et al., 2010; Holla et al., 2020; Murawska et al., 2021). We propose that loss of HDA-1 leads to increased histone turnover, which ultimately causes a

reduction in H3K9me3 density and HP1 condensates. We propose that temporary loss of HP1 binding allows PRC2 recruitment and nucleation of new H3K27me3 domains at regions previously marked by H3K9me3. Once established, PRC2 and H3K27me3 actively inhibit the DIM-5 H3K9 MTase. The opposite is observed in *Cryptococcus neoformans*. In *C. neoformans*, HP1 promotes aberrant H3K27 methylation at centromeric heterochromatin in the absence of Ccc1, a *C. neoformans*-specific PRC2 component (Dumesic et al., 2015). Ccc1 forms condensates that retain PRC2 at native sites, preventing PRC2 from forming weak interactions with HP1 condensates (S. Lee et al., 2023). In *N. crassa*, H3K27me3 and HP1 exhibit mutually exclusive localization patterns. This may reflect the differential properties of HP1 condensates in *N. crassa* and *C. neoformans*.

These results support what has been shown by others that in the absence of traditional heterochromatin components such as HP1 and HDA-1, PRC2 methylates constitutive heterochromatin domains (C.-H. Lee et al., 2018; Wang et al., 2017). We speculated several mechanisms or reasons for this activity. First, we suggest that PRC2 is recruited to these domains due to nucleosome density. Various remodeling factors can assist in creating an optimal nucleosome density for the recruitment of PRC2 (C.-H. Lee et al., 2018; X. Wang et al., 2017). In the absence of heterochromatin machinery, PRC2 might be interacting with non-canonical factors such as JARID2, a subunit of PRC2 in humans. JARID2 was shown to recruit PRC2 to AT-rich DNA via its "AT-rich interaction domain" (JARID) (Peng et al., 2009). Finally, PRC2 might be recruited to these domains to reinforce silencing in these regions to promote genome stability. Future studies need to focus on addressing why PRC2 is recruited to these domains in the absence of constitutive heterochromatin machinery.

We also reported that loss of HDA-1 leads to gradual epigenome instability, which might take hundreds of nuclear division cycles to accumulate. We know that the establishment and maintenance of epigenetics marks is complicated and involves multiple factors. Multiple feedback mechanisms might be acting together to prevent an instantaneous epigenome instability in the absence of constitutive heterochromatin machinery. In *Neurospora*, ASH1 deposited H3K36me3 colocalizes with H3K27me3 domains and drives gene repression (Bicocca et al., 2018). The gradual redistribution of H3K27me3 might also explain why we observed different redistribution patterns between HP1, HDA-1, and CDP-2 mutants. Some centromeres gained aberrant H3K27me3 while others did not. Since each of these strains was generated at a different time, we speculate that the variation in redistribution patterns might be due to the number of times the strain has been passaged. The redistribution of H3K27me3 to specific centromeres in each strain might also be sequence-specific. It has been reported that different strains have vastly different DNA sequences at their centromere in *Neurospora* (Smith et al., 2012). Future studies will explore if the recruitment of PRC2 to centromeres is random or sequence-specific. This will be accomplished by generating new HP1 and CDP-2 mutants and tracking the aberrant distribution over time. This will tell us if the targeting of PRC2 to centromeres is sequence-specific or random in each of these strains.

In mammals and *Schizosaccharomyces pombe*, disruption of HDA-1 led to a drastic reduction of H3K9me3 (Hohl et al., 2013; Sugiyama et al., 2007; Yamada et al., 2005). We noticed that in the absence of HDA-1, H3K9me3-enrichment is reduced in the *hda-1* mutant and replaced with H3K27me3. These results support previous studies that showed that deletion of HDA-1 in *Neurospora* led to a small reduction of H3K9me3 and DNA methylation levels only at small, less A:T-rich regions (Gessaman & Selker, 2017; Honda et al., 2012, 2016). These results suggest that

there might be multiple HDACs working together to regulate H3K9me3 in *Neurospora* (Seto & Yoshida, 2014; Smith et al., 2010).

The recruitment of HDACs in mammals is regulated by DNA methyltransferases (DNMTs) (Klose & Bird, 2006). The mammalian methyl-CpG-binding domain (MBD) MeCP2 was reported to recruit histone deacetylase one by binding to a region in the body of the *Dlx6* gene (Horike et al., 2005). These help to create a silent heterochromatin domain. We reported that cells lacking HDA-1 exhibited hyperacetylation at constitutive heterochromatin domains. Thus, increased hyperacetylation at heterochromatin domains leads to PRC2 recruitment. There might be a similar mechanism happening in mammals where HDACs are working together with DNA methyltransferases to ensure the tight regulation of silent chromatin states. DNMTs might serve to recruit proteins with MBDs, which in turn might serve as recruiting platforms for HDACs and other proteins. In the absence of HDA-1, PRC2 might be recruited to bind these MBDs, thus ensuring the maintenance of silent states in mammals.

In the absence of HDA-1, we reported an increase in acetylation at constitutive heterochromatin domains and a decrease in H3K27me3-marked domains. These results also support our previous hypothesis that different HDACs might be acting at different chromatin regions. HDA-1 might be regulating constitutive heterochromatin domains while a different HDAC might be regulating PRC2 function at facultative heterochromatin domains. It was recently reported that in *Neurospora*, a histone deacetylase complex (RPD3L) silences facultative heterochromatin (Mumford et al., 2024a). Loss of this histone deacetylase led to increased histone acetylation at PRC2-marked genes, with no changes observed in H3K27me3 levels. These results support our hypothesis that there might be different HDACs acting on constitutive and facultative heterochromatin to ensure the establishment and maintenance of silent chromatin states. Future

studies should focus on identifying other histone deacetylases that might be working with HDA-1 to regulate PRC2 function in *Neurospora crassa*.

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Figures

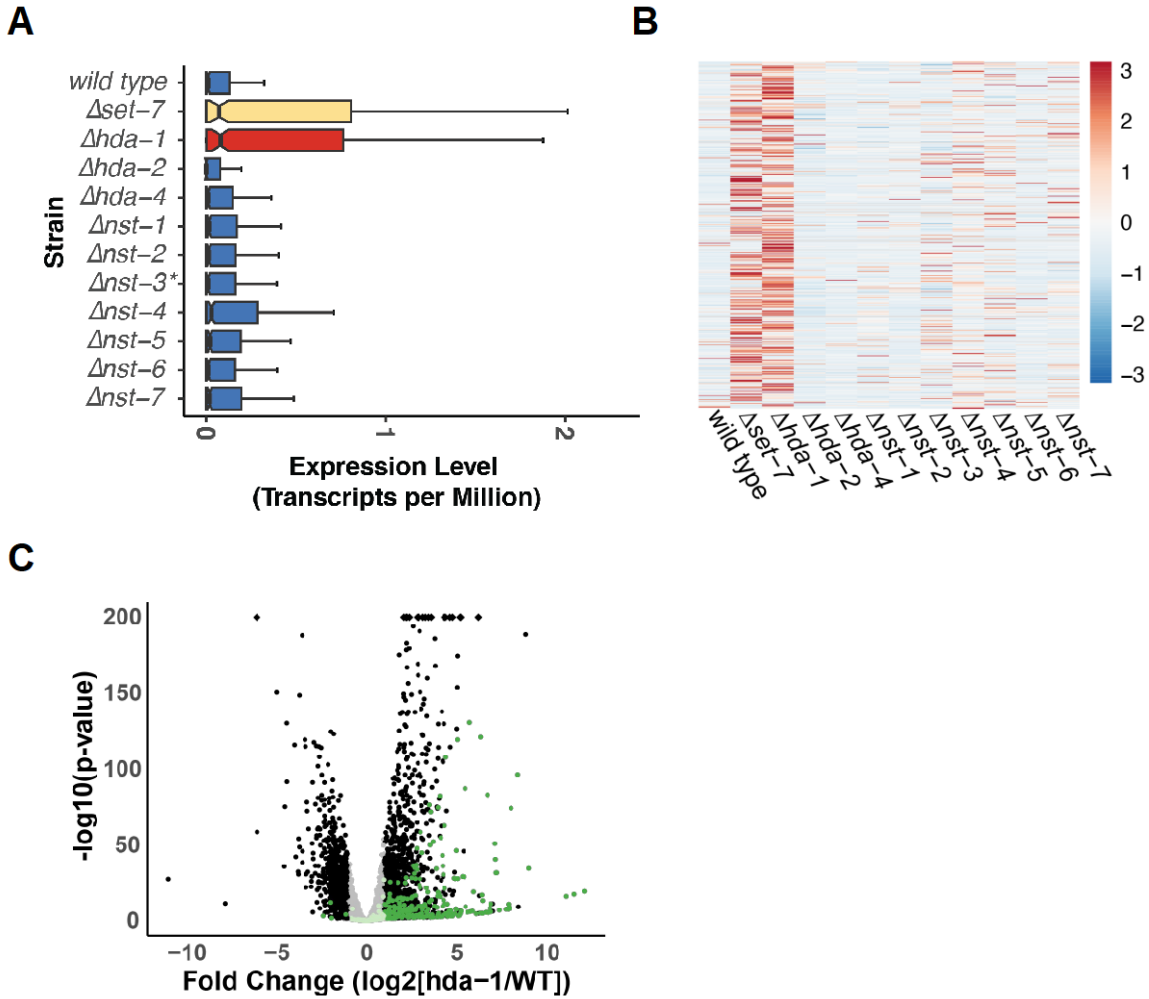


Figure 2.1: HDA-1 is required for the repression of PRC2-methylated genes. (A) The boxplot indicates the average level of expression for all PRC2-target genes (n= 642) for the indicated strains. The x-axis values correspond to log₂-transformed transcripts per million (TPM) read counts for PRC2-target genes for each strain. The line represents the median expression value. The notch indicates the 95% confidence interval. (B) The heat map shows the relative expression of each PRC2-target gene (n= 642) in the indicated strains. (C) The volcano plot illustrates the differential expression of genes between the wild-type and the $\Delta hda-1$ mutant. Genes with a p-value $< X$ and a ± 1.5 -fold change are indicated by dark green points for PRC2-methylated genes and black points for all other genes. Genes that fail to meet fold-change and significance thresholds are plotted in green (PRC2-target genes) or grey (all other genes).

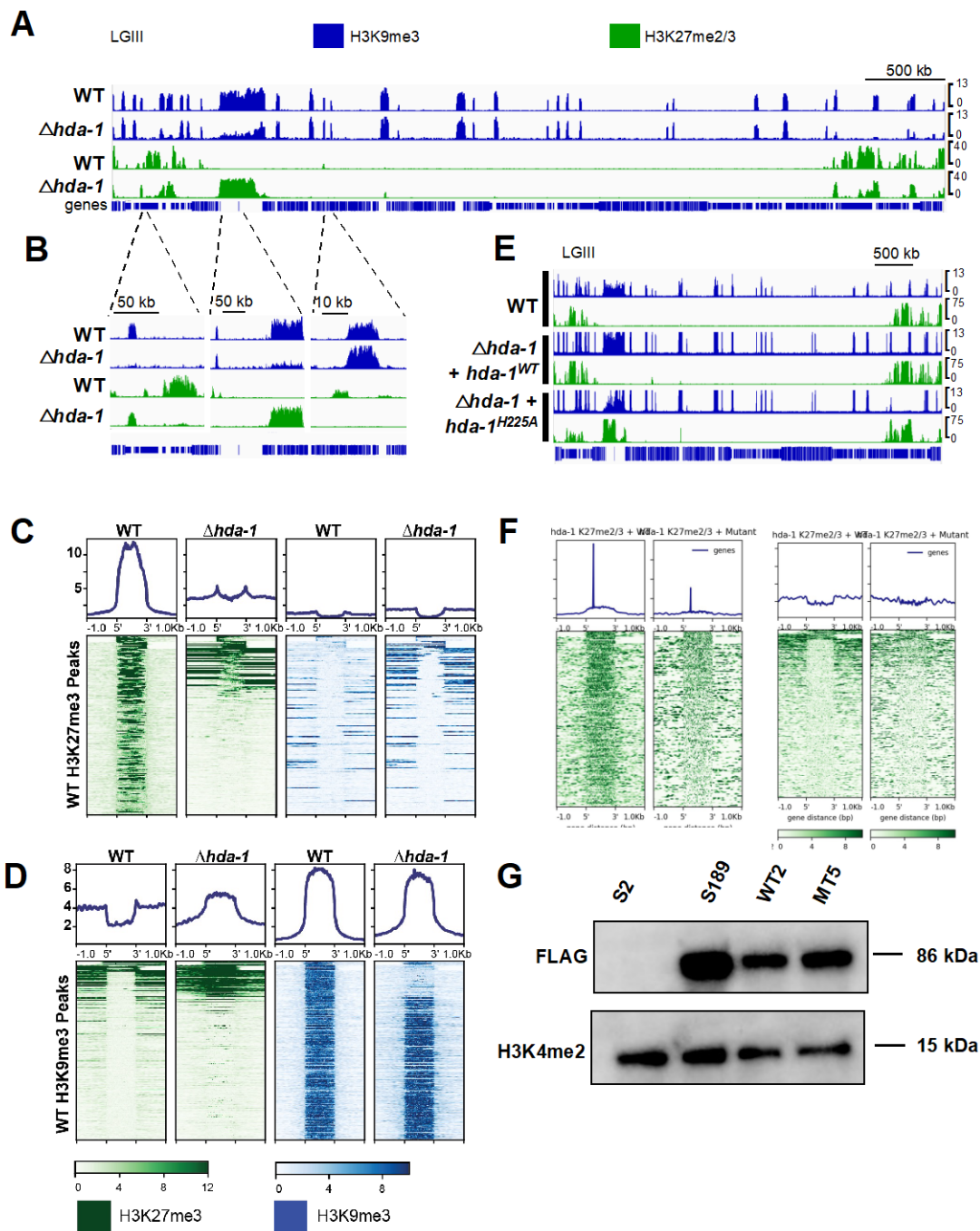


Figure 2.2: H3K27me3 is aberrantly localized to heterochromatin in the $\Delta hda-1$ mutant. (A) The genome browser tracks show the distribution of H3K9me3 (blue) and H3K27me3 (green) across Linkage Group III. Enrichment was determined by CUT&RUN. The scale bar corresponds to 500 kilobase pairs (kb). (B) A magnified genome browser view of three regions on LGIII is shown. The left panel shows a region in which H3K27me3 is lost from a typical PRC2-methylated domain in the $\Delta hda-1$ strain. The middle panel shows a region that loses H3K9me3 and gains H3K27me3 in the $\Delta hda-1$ strain. The right panel shows an H3K9me3 region that does not gain H3K27me3 in the $\Delta hda-1$ strain. (C) The heatmap depicts H3K27me3 (green) or H3K9me3 (blue) enrichment across all WT H3K27me3 peaks for wild type (WT) or $\Delta hda-1$, as indicated. The first 14 rows of the heatmap correspond to sub-telomeric peaks enriched both H3K27me3 and H3K9me3. The remaining rows are sorted by H3K27me3 enrichment levels in $\Delta hda-1$. Each peak is scaled to normalize peak sizes to 100%, and 1000 base pairs is shown on the 5' or 3' side of each peak. (D) The heatmap depicts H3K27me3 (green) or H3K9me3 (blue) enrichment across all WT H3K37me3 peaks for wild type (WT) or $\Delta hda-1$, as indicated. Rows are ordered as in C. (E) The distribution of H3K9me3 (blue) and H3K27me3 (green) determined by CUT&RUN was plotted across Linkage Group III using the Integrated Genome Viewer for wildtype (WT) and for an $\Delta hda-1$ strain transformed with a wild type *hda-1-3xflag* construct or an *hda-1^{h225a}-3xflag* construct, as indicated. (F) Heatmaps showing H3K27me3 peaks across all PRC2-target genes in wildtype, WT *hda-1-3xflag* and $\Delta hda-1^{h225a}-*3xflag* strains. (G) Both WT *hda-1-3xflag* and $\Delta hda-1^{h225a}-*3xflag* fusion proteins are expressed at similar levels. Total protein extracts were analyzed by Western blotting using an anti-FLAG antibody. H3K4me2 was used as a loading control for the indicated strains.$$

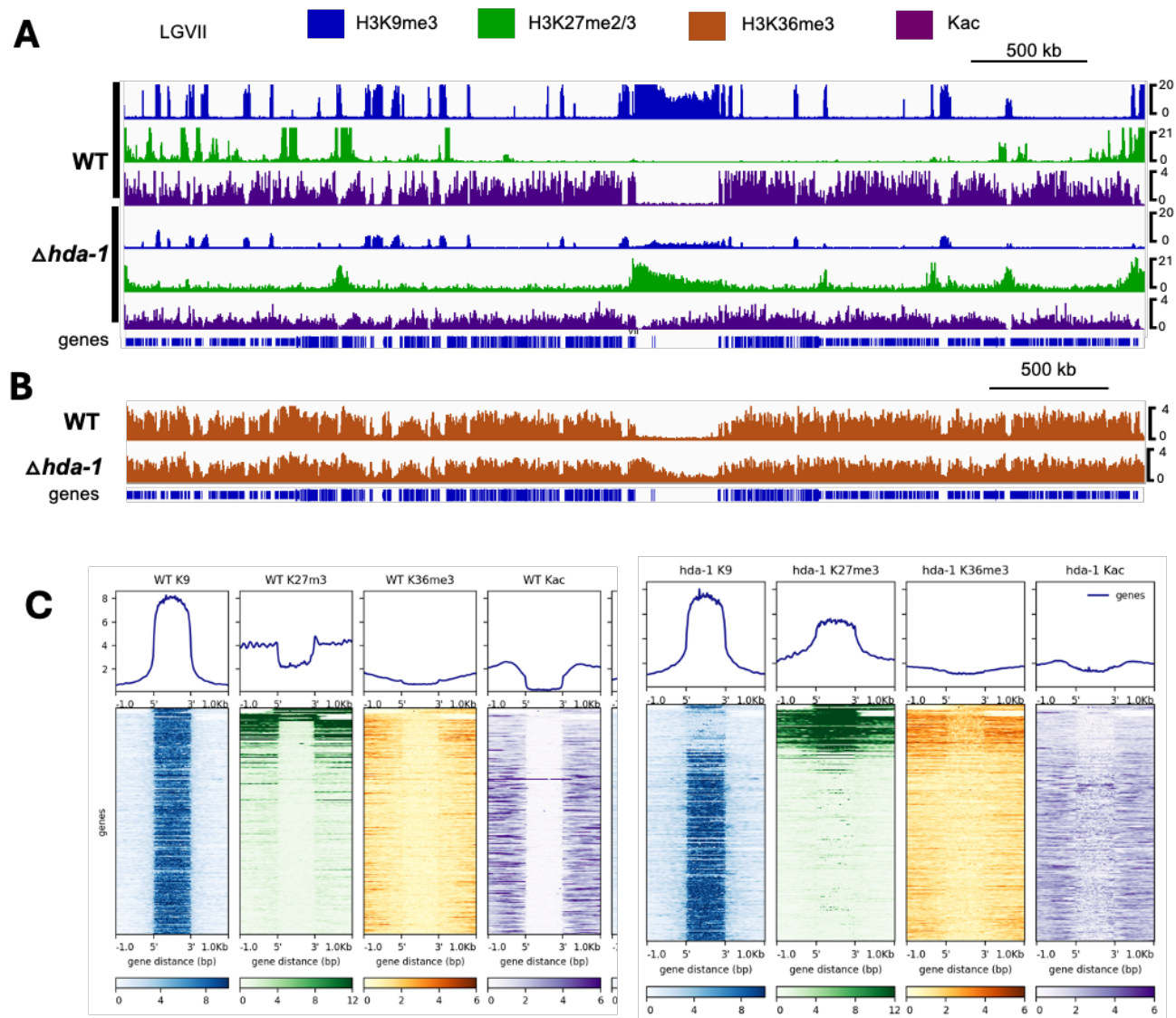


Figure 2.3: Acetylation and H3K36me3 patterns are altered in $\Delta hda-1$. (A) The genome browser tracks show the H3K9me3 (blue), H3K27me3 (green), and pan-acetyl lysine (Kac; purple) across Linkage Group VII. Enrichment was determined by ChIP-seq. The scale bar corresponds to 500 kilobase pairs (kb). (B) The distribution of H3K36me3, determined by ChIP-seq, is shown as in A. (C) The heatmap depicts ChIP-seq enrichment of H3K9me3 (blue), H3K27me3 (green), H3K36me3 (orange), and pan-acetyl lysine (H3Kac; purple) for wild-type (WT) or $\Delta hda-1$, as indicated, across all wild-type H3K9me3 peaks. The first 14 rows of the heatmap correspond to sub-telomeric peaks enriched in both H3K27me3 and H3K9me3. The remaining rows are sorted by H3K27me3 enrichment levels in the $\Delta hda-1$ strain. Each peak is scaled to normalize peak sizes to 100%, and 1000 base pairs is shown on the 5' or 3' side of each peak.

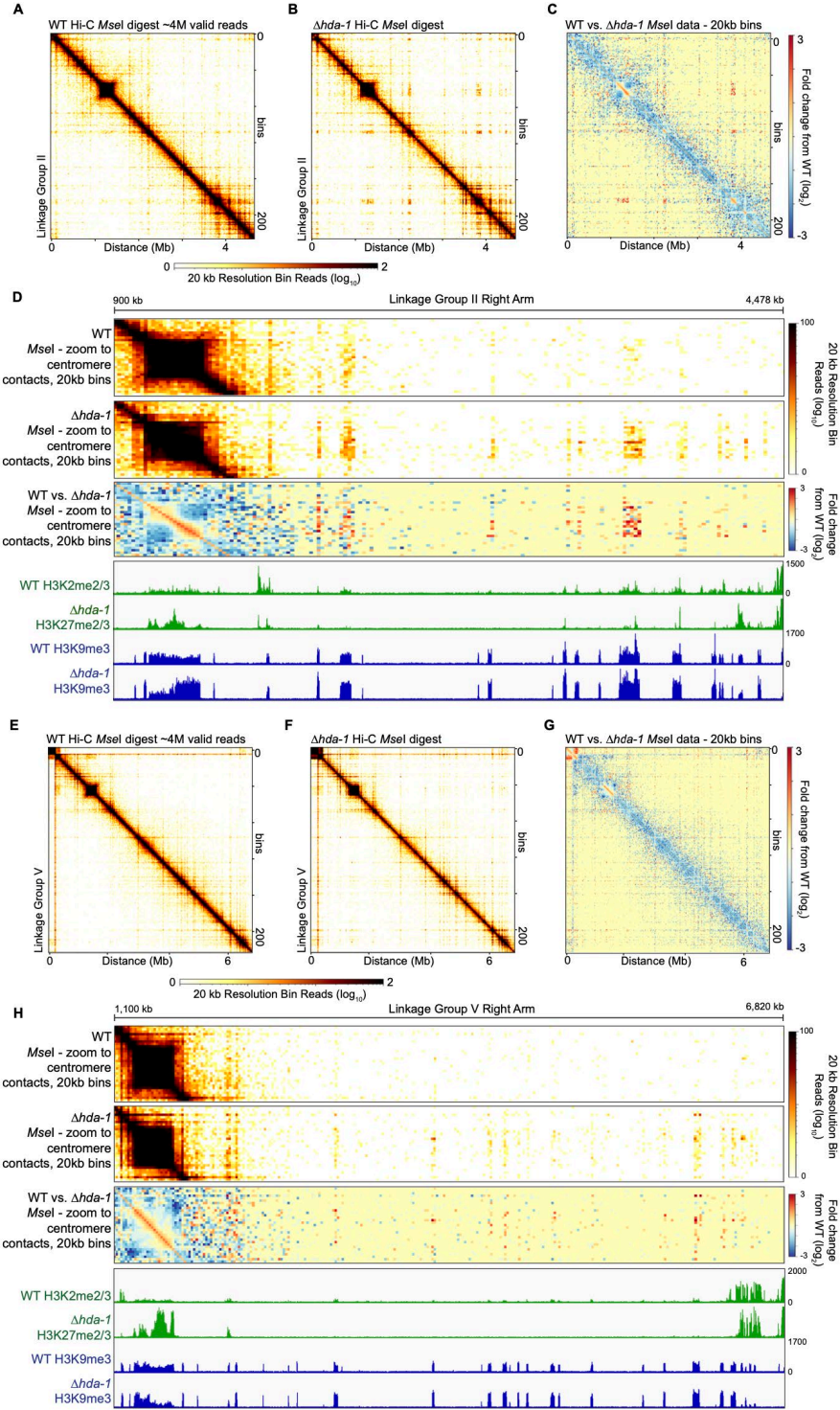


Figure 2.4: Inter-heterochromatic region contact gains and variable H3K27me2/3 enrichment occur in a $\Delta hda-1$ strain. Heterochromatin-specific (*MseI*) *in situ* Hi-C and H3K27me2/3 or H3K9me3 ChIP-seq of a WT strain or a $\Delta hda-1$ strain, NKL9, are displayed across two Linkage Groups (LG), LG II (**A-D**) or LG V (**E-H**). Contact probability heatmaps of raw *in situ* Hi-C reads at 20 kb resolution of a WT strain (**A, E**) or the NKL9 $\Delta hda-1$ strain (**B, F**) are shown; the heatmap scalebar is below, while the genomic distance is indicated on the x-axis and the number of bins indicated on the right. (**C, G**) The comparison heatmaps of the contact probability change ($\log_2$) between WT and $\Delta hda-1$ strains are shown; the heatmap scalebar is to the right. The purple line shows the centromeric region highlighted in panels D and H. (**D, H**) Enhanced contact probability heatmaps of a WT strain (top), the $\Delta hda-1$ NKL9 strain (middle), and the change in contact probability between the WT and $\Delta hda-1$ strains (bottom) between the centromeres and right chromosomal arm are displayed at 10 kb resolution. IGV images of H3K27me2/3 (green) and H3K9me3 (blue) ChIP-seq tracks of WT and $\Delta hda-1$ strains of the same region are shown below. Distances of LG II (panel **D**) and LG V (panel **H**) are shown above.

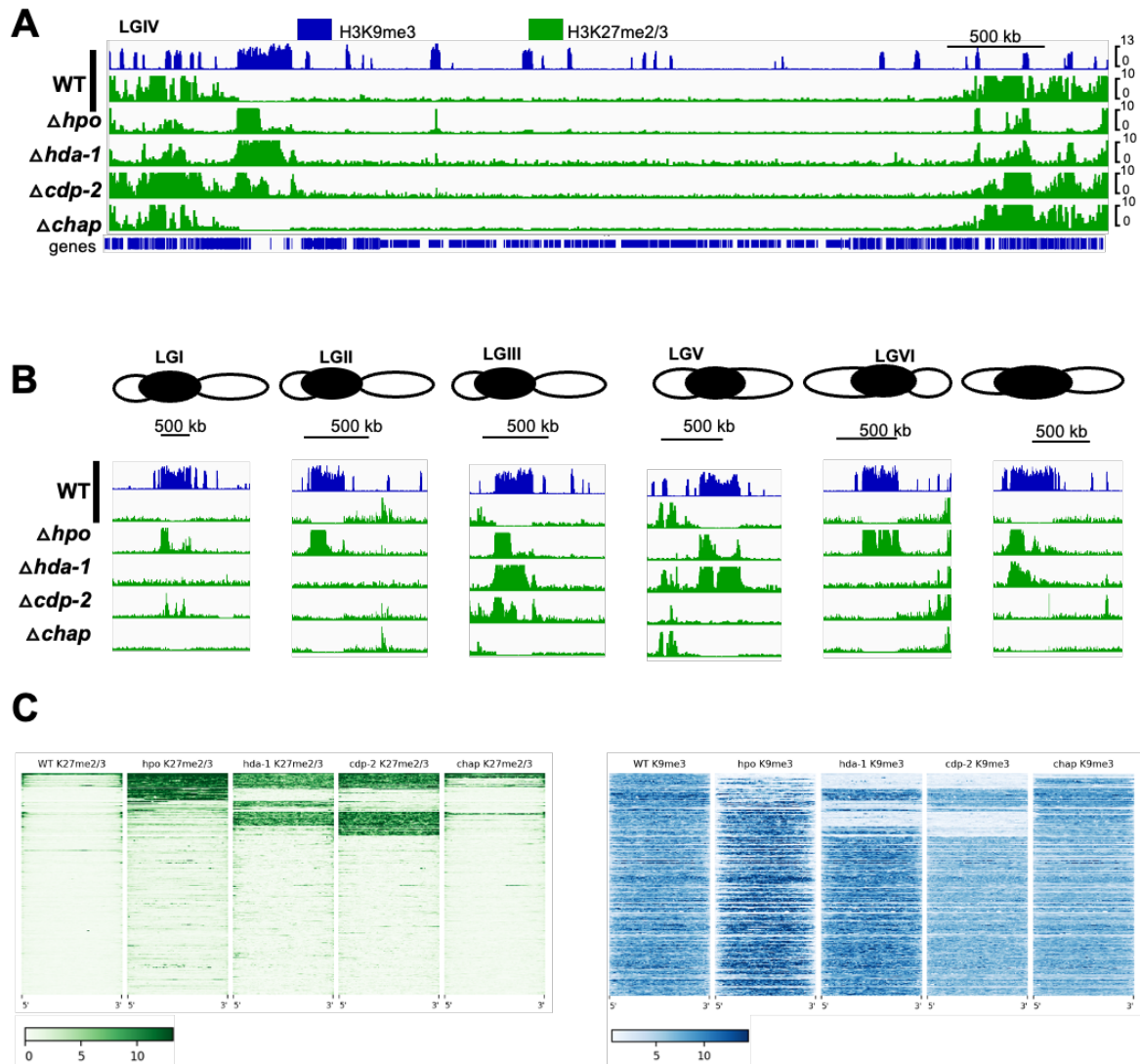
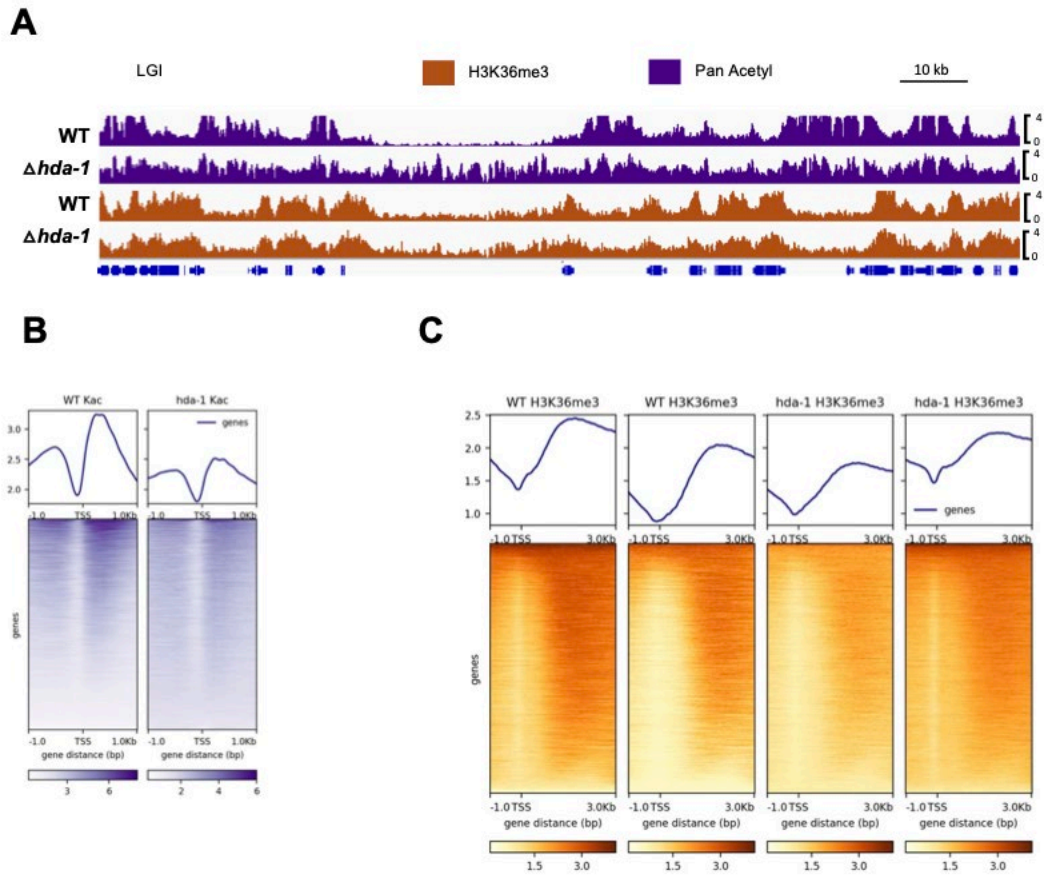


Figure 2.5: Other CHAP components are also required for normal H3K27me3 patterns. (A) The genome browser tracks show the distribution of H3K9me3 (blue) and H3K27me2/3 (green) across Linkage Group IV for the indicated strains. Enrichment was determined by CUT&RUN. The scale bar corresponds to 500 kilobase pairs (kb). (B) A magnified genome browser view of all seven centromeres is shown for the indicated strains (I-VII). (C) Heat maps revealed a distinct pattern of H3K27me2/3 enrichment in each strain. The heatmaps show H3K27me2/3 (green) and H3K9me3 (blue) enrichment for the indicated strains across almost all wild type H3K9me3 domains. Not shown in the heat map are 14 domains adjacent to each telomere, which are enriched for both H3K9me3 and H3K27me2/3 in all strains.

Supplementary Figures



Supplementary Figure 2.1. Acetylation heatmap across the TSS of all genes to show euchromatin is not affected. (A) ChIP-seq data showing H3K36me3 (orange) and H3Kac (purple) enrichment in wildtype (WT) and in the *hda-1* mutant. Heatmap data showing acetylation **(B)** and H3K36me3 **(C)** enrichment across the TSS of all genes.

Tables

Table 3.1. Strains used in this study

Strain	Genotype	Source
S2	Wild type	This study
S297	<i>hda-1::hph</i>	This study
S181	<i>hpo::hph</i>	This study
S188	<i>hda-1::hph his-3-</i>	This study
S298	<i>cdp-2::hph</i>	This study
S299	<i>CHAP::hph</i>	This study
FAE512	Δ hda-1 + WT hda-1-3xflag	This study
FAE525	Δ hda-1 + MUT hda-1-3xflag	This study
FAE457	New Δ hda-1 SM1+SM2	This study

CHAPTER 3

POSITIVE FEEDBACK BETWEEN H3K27ME3, ISW, AND H3K36ME3 PROMOTES
MITOTIC INHERITANCE OF H3K27ME3

Felicia Ebot-Ojong, Eduardo Torres and Zachary Lewis. To be submitted to *Genetics*.

Abstract

Nucleosomes, the fundamental units of chromatin, are made up of 147-150 base pairs of DNA wrapped around histone cores. The histones' tails are modified with several post-translational modifications (PTMs), such as acetylation and methylation. These PTMs play a key role in gene regulation without changing the DNA sequence of the genome. Some of these modifications can be established (initial deposition on new histones) and maintained (passed down to the next generation). The establishment and maintenance of these modifications occur simultaneously in the genome, which present significant experimental challenges in studying how these marks are assembled and maintained over time. To overcome this challenge, we developed an experimental system to distinguish between facultative heterochromatin establishment and maintenance. Using *Neurospora crassa*, we showed that ectopic targeting of the PRC2 subunits SUZ12 or EED is sufficient to induce artificial domains of histone H3 lysine 27 methylation (H3K27me), a well-conserved marker of heterochromatin. Furthermore, we found that H3K27me3 is sufficient to direct H3 lysine 36 methylation (H3K36me3) in the artificial domain. The recruitment of H3K36me3 relies on ISWI, an ATP-dependent chromatin remodeling enzyme. We also show that ISWI enhances mitotic inheritance of H3K27me3 in *Neurospora crassa*. Our findings provide a new model in *Neurospora* where PRC2 activity and H3K36me3 work together in a positive feedback loop to maintain “silent” heterochromatin states.

Introduction

The field of epigenetics is focused on exploring how heritable phenotypes persist through mitosis and meiosis, independent of changes to the genomic code (Fukuda et al., 2023; Goldberg et al., 2007; Margueron & Reinberg, 2010; Ospelt, 2022; Ptashne, 2007). In eukaryotic cells,

DNA is wrapped around core histone octamers to form a nucleosome, the basic unit of chromatin (Bernardi, 2021; Kornberg, 1974). Each nucleosome is marked with chemical modifications such as methylation and acetylation (Kouzarides, 2007; Millán-Zambrano et al., 2022; Ramazi & Zahiri, 2021). These post-translational modifications carry crucial information that regulate genome functions like transcription, DNA repair, and DNA replication. They also control critical cellular processes such as Polycomb silencing and X-chromosome inactivation (Bannister & Kouzarides, 2011; Cao et al., 2002; Fischle et al., 2003; Millán-Zambrano et al., 2022; Plath et al., 2003; Y. Zhang et al., 2021). Despite the importance of these modifications, the mechanisms by which histone modifications are inherited during and after DNA replication is poorly understood.

During replication, chromatin is unfolded, and the old nucleosomes containing PMTs are diluted by a factor of 2 during S-phase (Escobar et al., 2021; Reverón-Gómez et al., 2018b; Stewart-Morgan et al., 2020). After random distribution of old histones to the daughter strands, newly synthesized histones are deposited to restore the normal nucleosome occupancy (Escobar et al., 2019; Schlissel & Rine, 2019). However, only some of these histone modifications are reestablished after replication. Studies using proteomic analyses of pulse-labeled histones showed that acetylation and methylation marks on parental histones were recycled to sister chromatids during DNA replication (Alabert et al., 2015; Gan et al., 2018; Reverón-Gómez et al., 2018b; Scharf et al., 2009; M. Xu et al., 2011; C. Yu et al., 2018). Additional genetic studies in mammals, flies, worms, and yeast have shown that genes marked with H3K9me3 and H3K27me3 are inherited through cell division (Audergon et al., 2015; Coleman & Struhl, 2017; Gaydos et al., 2014; Hansen et al., 2008; Hathaway et al., 2012; Laprell et al., 2017; Michieletto et al., 2018; Ragunathan et al., 2015; Ramachandran & Henikoff, 2015). These chromatin states

are maintained by chromatin modifying enzymes with ‘read’ and ‘write’ activity. This ensures that the modifications are reestablished following replication (Hyun et al., 2017; Margueron et al., 2009; Uckelmann & Davidovich, 2021b). An example of such a histone modification is H3K27me3. H3K27me3 marks repressed heterochromatin and is deposited by the enzymatic complex Polycomb Repressive Complex 2 (PRC2) (Margueron & Reinberg, 2011a). The SET domain containing the protein Ezh2 deposits H3K27 methylation on nucleosomes, and another subunit, EED, binds the repressive methyl-lysine marks and ensures its propagation. Other factors that influence mitotic stability of chromatin states are poorly understood.

A “classic model” for H3K27me3 establishment and maintenance has been described in the model system *Drosophila melanogaster*. In *Drosophila*, *cis*-acting DNA sequences called Polycomb Response Elements (PREs) are necessary for both H3K27me3 establishment and maintenance (Alecki et al., 2020; Coleman & Struhl, 2017, 2017; Czermin et al., 2002b; Ghotbi et al., 2021; Iovino et al., 2013; Kassis et al., 2017b; Laprell et al., 2017; Tolhuis et al., 2006). PREs ensure proper recruitment of Polycomb group (PcG) proteins to prevent inappropriate activation of Hox genes (Bredesen & Rehmsmeier, 2019; Duncan, 1982; Jürgens, 1985; Struhl, 1981). In the model yeast *Schizosaccharomyces pombe*, targeting the H3K9 methyltransferase (Clr4) was sufficient to establish an artificial domain of H3K9me3 (Ragunathan et al., 2015). The artificial H3K9me2/3 domains were maintained for several generations through the ‘read–write’ function of the *Clr4* methyltransferase but only in the absence of the demethylase *Epe1*. The establishment and maintenance of PRC2 is more complicated than the simplistic model described in *Drosophila* and yeast. In higher eukaryotes and *Drosophila*, recruitment of PRC1 or PRC2 is sufficient to establish heterochromatin domains. These examples highlight the

complex mechanisms that are involved in the epigenetic establishment and maintenance of silenced heterochromatic states.

Very little is known about the mechanisms that regulate H3K27me3 establishment and maintenance in *Neurospora*. The core subunits of the PRC2 complex are both structurally and functionally conserved between plants, animals, and fungi. The core subunits that make up the PRC2 complex in *Neurospora* are SET-7, EED, and SUZ12 (Brickner, 2023; Guo et al., 2021; Uckelmann & Davidovich, 2021b; J.-R. Yu et al., 2019). H3K27me3 is deposited by the catalytic subunit SET-7 (Jamieson et al., 2013b). In *Neurospora*, the fourth subunit of PRC2, CAC-3/NPF, plays a role in enhancing PRC2 function (Glancy et al., 2021; Jamieson et al., 2013a). The PREs known to recruit PRC2 in *Drosophila* have not been identified in *Neurospora* or any other fungi. Telomere repeats are sufficient to induce an ectopic H3K27me2/3 domain (Jamieson et al., 2018), and the methylation of H3K27 is position-dependent. *Neurospora* and other fungi lack the PRC1 complex identified in plants and animals (Jamieson et al., 2013b). Normal patterns of H3K27me3 in *Neurospora* are regulated by the PRC2 Accessory Subunit (PAS) ATP-dependent chromatin remodeling enzymes ISWI and ACF and histone variant H2A.Z (Courtney et al., 2020; Jamieson et al., 2013; Kamei et al., 2021; McNaught et al., 2020; Wiles et al., 2022). IMITATION SWITCH (ISWI) complexes are ATP-dependent chromatin remodeling enzymes. One of the ISWI complexes is the ATP-Utilizing Chromatin Assembly and Remodeling Factor (ACF) complex, which contains ACF1 and ISWI. This complex has been linked to the regulation of PRC2 and PRC2-targeted domains in *Drosophila* and other systems (Deuring et al., 2000; Fyodorov & Kadonaga, 2002; Scacchetti et al., 2018; Stopka & Skoultschi, 2003). *N. crassa* homologs of ISWI are required for the repression of a subset of PRC2-targeted genes (Kamei et al., 2021; Wiles et al., 2022a). In *Neurospora*, the ISWI homolog, referred to

simply as ISW, is essential for maintaining proper patterns of histone modifications, specifically H3K27me3 and H3K36me3 (Kamei et al., 2021). Despite recent advances, how these factors control the establishment and/or maintenance of PRC2-repressed chromatin in *Neurospora* and other systems remains unknown.

We developed an experimental system to separate establishment and maintenance. This genetic system to enable chemical control of facultative heterochromatin formation was developed by fusing one of three PRC2 subunits (SUZ12, EED, or CAC3) to the bacterial tetracycline repressor (TetR) protein. In the absence of tetracycline, the tetR-PRC2 fusion binds to an array of tet operator repeats (tetO) and deposits H3K27me3. Adding tetracycline leads to the release of the TetR-PRC2 complex from the DNA, enabling us to observe how this release affects the stability and integrity of the heterochromatic region. We showed that SUZ12 and EED are sufficient to establish an ectopic domain of H3K27me3 in the absence of sequence-specific recruitment. The ectopic domain of H3K27me3 was weakly maintained once the TetR-PRC2 complex dissociated from the DNA by the addition of tetracycline. We also reported that H3K27me3 is sufficient to recruit another histone modification, H3K36me3. The PRC2-dependent recruitment of H3K36me3 to the tetO array is dependent on the activity of the ATP-dependent chromatin remodeling enzyme ISWI. In the absence of ISWI, H3K27me3 is poorly inherited, suggesting a new role of H3K36me3 and ISWI in the mitotic stability of H3K27me3-repressed chromatin domains.

Materials and Methods

Strains and growth conditions

Vegetative cultures were initiated from macroconidia and cultivated in Vogel's minimal medium with 1.5% sucrose and shaken at 32°C for 18 hours unless stated otherwise in the results (Davis & de Serres 1970). Tetracycline was added to the growth medium at a final concentration of 5 µg/ml where indicated. The strains used as controls were constructed using standard *Neurospora* protocols described in Colot et al. (2006) and Davis & de Serres (1970). All strains used in this study are listed in Table 3.2, and the primers used to build our reporter strains are listed in Table 3.1.

Plasmid construction

We used Gibson cloning to generate plasmids containing three different transgenes fused to a tetR^{OFF}GFP module (Table 3.1). The tetR^{OFF}GFP plasmid was gifted to us by Eugene Gladyshev (Nguyen & Gladyshev, 2020). Plasmids were assembled as described in Gibson et al. (2009). Each plasmid encodes a PRC2 component (EED, SUZ-12, and CAC3) (Jamieson et al., 2013a) as illustrated in (Figure 3.1B). The constructed plasmids were validated using Sanger sequencing and Plasmidsaurus (Supplementary Figure 3.1A). Next, the validated plasmids were digested with the DraI restriction enzyme, cleaned with serapure beads (Polysciences, Cat #86057-3), and transformed into our host strain (S542).

Strain construction

To construct our tetR-GFP-PRC2 strains, we used a host strain containing 240 tetO sites (S542) that was gifted to us by Eugene Gladyshev. We validated this strain by extracting genomic DNA and using primers to amplify across the *his-3* insertion site (Table 3.2). The linearized plasmids were integrated into the *csr-1* locus of *N. crassa* strain S542. A conidia suspension of about conidia (2.0×10^9 /ml) was prepared in 100 µl of deionized water for the transformation. A solution (3-5 µl) containing 2-5 µg DNA of the linearized plasmid was mixed

with 50 µl of the conidial suspension and incubated on ice for 15 min. 40 µl of the mixture was transferred to an electroporator BTX Electro Cell Manipulation 600, Genetronics) and shocked by using the standard settings for electroporating conidia (1500 V, 600 Ohms, and 25 µF). After the electroschock, 1 ml of cold 1M sorbitol was added to dilute the electroporated conidia/DNA suspension and incubated while shaking at 200 RPM at 30°C for 1-2 hours. 200 µl of transformation solution was spread on agar medium plates containing Cyclosporin A (Sigma #30024) selection: 5 µg/ml final concentration from 2.5 mg/ml stock (500X) diluted in ethanol. Colonies resistant to Cyclosporin A were isolated and tested by PCR using the *csr-1* genotyping primers to determine whether the *csr-1* gene was replaced and if the transgene (tetR-GFP-SUZ12, tetR-GFP-EED or tetR-GFP-CAC-3) was integrated into the *csr-1* locus (Supplementary Figure 3.1B).

To delete *isw* in strain FAE275 (*his-3::tetO Δcsr-1::tetR-gfp-suz12*), we used the *isw* KO primers listed in Table 3.1 to amplify the *isw* knock-out cassette from an *isw* mutant strain S614. We introduced the *isw* KO cassette into strain FAE275 (*his-3::tetO Δcsr-1::tetR-gfp-suz12*) by electroporation and selected for hygromycin-resistant colonies as described (Ninomiya et al., 2004). HYG-resistant primary transformants were tested by PCR using primers listed in Table 3.1 to confirm deletion of the *isw* gene. All PCR amplifications were performed using LaTAQ (Cat. # RR002A) and Phusion polymerase (Cat. # M0530S) following the manufacturer's protocol.

Chromatin immunoprecipitation (ChIP-seq)

ChIP-seq was performed as previously described (Ferraro & Lewis, 2018) with the following antibodies: H3K27me2/3 (Active Motif Cat# 39535); H3K27me3 (Abcam Cat#

ab6002); H3K36me3 (Abcam Cat# ab9050); GFP (Abcam Cat # ab290). Libraries were created and analyzed as previously described (Ferraro & Lewis, 2018). The libraries were submitted for sequencing at the Georgia Genomics Facility at the University of Georgia on a NextSeq 500 instrument

ChIP-qPCR

The primers used for quantitative PCR (qPCR) are listed in Table 3.1. Following ChIP-seq, the DNA was diluted in dH₂O in a 1:50 ratio for the input samples and 1:10 ratio for samples immunoprecipitated with the different antibodies tested. For PCR setup, primers were mixed with qPCR SYBR Green Mix (Cat # 4309155), and 1 µl of the diluted ChIP DNA was added into each well. Samples were run on the qPCR machine based on the manufacturer's instructions.

Data analysis

To process the ChIP-seq data, short reads (less than 20 bp) and adapter sequences were eliminated using TrimGalore (version 0.6.7). The trimmed Illumina reads were then aligned to the latest *N. crassa* NC12 genome assembly from NCBI (accession # GCA_000182925.2) using BWA (version 0.7.17) with the mem algorithm, which randomly assigns multimapped reads to a single position. The resulting files were sorted and indexed with SAMtools (version 1.16.1). For visualizing the distribution of mapped reads, read counts were calculated for each 10-bp window across the genome using deeptools (Ramírez et al., 2016), bamCoverage using the 'BPM' normalization option, and the data were visualized with the Integrated Genome Viewer (version 2.3.72) (Robinson et al., 2011) .

Results

Construction of an inducible system to separate heterochromatin establishment and maintenance

Histone-modifying enzymes can be targeted to establish ectopically silent chromatin domains (Chien et al., 1993; Kagansky et al., 2009; Ragunathan et al., 2015). We tested if targeting PRC2 was sufficient to establish an artificial H3K27me₃ domain by using the TetO/TetR system. This system in *Neurospora* was initially developed and described by Nguyen and Gladyshev (2020). We fused different PRC2 subunits (SUZ12, EED, and CAC3) to a TetR-GFP module. This system offers a versatile platform for the inducible control of gene expression in a simple system such as *Neurospora crassa*. In this system, the TetR DNA-binding domain interacts with its cognate DNA binding sequence located at a target locus (Figure 3.1A). The recruitment of TetR to this locus can be inhibited by the addition of tetracycline, a feature of the TetR-off system (Gossen & Bujard, 1992). We generated three different TetO/TetR strains where TetR-SUZ12, TetR-EED, and TetR-CAC3 transgenes were introduced into the *csr-1* locus of the host strain (S542) containing 240 tetO repeats (Figure 3.1B). We grew these strains in the presence and absence of tetracycline to determine how artificial H3K27me₃ domains are established and maintained in *Neurospora crassa*.

Artificial targeting of PRC2 establishes an ectopic domain of H3K27me₃

First, we tested if the tethering of a PRC2 subunit was sufficient to establish an artificial domain of H3K27 me_{2/3} at the tet-O array. We used ChIP-qPCR to quantify H3K27me_{2/3} levels at a *his-3* flank region. The strains with the TetR-GFP-EED and TetR-GFP-SUZ12 had increased H3K27me_{2/3} enrichment at the *his-3* flank region compared to our control strain (S542) (Figure 3.1C). However, no enrichment of H3K27me_{2/3} was observed in strains expressing TetR-GFP-CAC3 (Figure 3.1C).

Next, we used ChIP-seq to confirm that H3K27me3 was established specifically at the tetO array. Our ChIP-seq analysis revealed significant enrichment of H3K27me3 at the tetO array in strains containing TetR-GFP-SUZ12 and TetR-GFP-EED fusions. In contrast, H3K27me3 was not enriched in a control strain that only contained the tetO array (Figure 3.1D). Expression of the tetR-GFP-SUZ12 or tetR-GFP-EED fusion had no effect on H3K27me3 patterns at native PRC2-methylated domains. Multiple independent transformants showed similar results (Supplementary Figure 3.2). Based on these results, we conclude that artificial targeting of SUZ12 and EED can recruit the Polycomb Repressive Complex 2 (PRC2) components to induce an ectopic H3K27me3 at the tetO array. We did not see any H3K27me3 enrichment at the tetO array in strains containing the fusion protein of TetR-GFP-CAC3.

The artificial H3K27me3 domain spreads beyond the nucleation site

PRC2-target domains in *Neurospora* and other systems are large and cover multiple genes (Jamieson et al., 2013a). Since we confirmed that an ectopic H3K27me3 domain was established in our TetR fusion strains, we wanted to investigate whether the artificial H3K27 methylation domain spreads beyond the nucleation site. We obtained better H3K27me3 enrichment strains harboring the tetR-GFP-SUZ12 construct and therefore used these strains for rest of the study. In this strain, we observed the spread of H3K27me3 beyond the tet-O array into neighboring genes (Figure 3.2A). We compared H3K27me3 enrichment data at the tet-O array across our original his-3::tetO tetR-SUZ12 primary transformant strain and data from different passages of that same strain. We saw that H3K27me3 spreads to cover multiple genes and large gene domains. These findings support the PRC2 ‘write and read’ mechanism (Uckelmann & Davidovich, 2021b). According to this mechanism, once H3K27me3 is established, EED recognizes and binds to the newly deposited mark. This binding event stimulates the catalytic

activity of SET-7, thereby promoting the spread of H3K27me3 to adjacent nucleosomes (J.-R. Yu et al., 2019).

H3K27me3 is lost after several rounds of cell division following the addition of tetracycline

Next, we wanted to investigate whether the artificial H3K27me3 domain is maintained upon the release of the TetR-GFP-SUZ12 fusion protein. We grew our *his-3::tetO tetR-GFP-SUZ12* strain in liquid Vogel's media and liquid Vogel's media supplemented with tetracycline for 6, 12, 24, or 36 hours (Figure 3.3A). Adding tetracycline to the media should lead to the dissociation of the TetR-GFP-SUZ12 fusion protein. We then performed ChIP-seq to look at H3K27me3 levels at the tetO array. We saw that the addition of tetracycline led to a notable loss of H3K27me3, as evidenced by the significant reduction of H3K27me3 levels at the artificial domain from strains grown with tetracycline for 6 hours vs those grown for 36 hours (Figure 3.3B). After 36 hours of tetracycline treatment, the H3K27me3 levels were significantly reduced but remained above background levels. We are not exactly sure how many cell division cycles are completed by 36 hours, but we conservatively estimate that by 36 hours, the cells have gone through approximately 12 rounds of cell division (3 hours/nuclear division). Thus, if H3K27me3 is passively diluted each nuclear division cycle, we expect to see a >4000-fold dilution by 36 hours in tetracycline in the absence of *in vivo* maintenance. These results suggest that H3K27me3 at the tetO array is weakly maintained in the absence of the tethered TetR-GFP-SUZ12 fusion protein.

Targeting H3K27me3 is sufficient to recruit H3K36me3

ASH1 (NCU01932) is involved in catalyzing H3K36me_{2/3} in *Neurospora*. The activity of ASH1 has been linked to PRC2 regulation (Bicocca et al., 2018). Most genes marked by ASH1-mediated H3K36 methylation were also co-modified with H3K27me_{2/3}. These results suggested a mechanistic pathway where ASH1-mediated H3K36 methylation was sufficient to recruit PRC2. This prompted us to investigate whether H3K27me₃ can reciprocally influence H3K36 methylation at an artificial chromatin domain. We looked at H3K36me₃ enrichment at the tetO array of our *his-3::tetO tetR-gfp-suz12* strain. Our ChIP-seq analysis revealed that H3K27me₃ was sufficient to target H3K36me₃ to the artificial domain (Figure 3.4A). H3K36me₃ peaks were detected at the artificial tet-O array, and these align with induced H3K27me₃ in the *his-3::tetO tetR-gfp-suz12* strain. Our control strain (*his-3::tetO tetR-GFP*) had no detectable H3K27me₃ or H3K36me₃ peaks at the tet-O array. These results show that H3K27me₃ is sufficient to recruit H3K36me machinery.

The recruitment of H3K36me₃ to targeted domains is dependent on ISW

Next, we wanted to test if we can use our *his-3::tetO tetR-GFP-SUZ12* strain to identify any chromatin regulators that might be involved in the establishment or maintenance of the silent H3K27me₃ domains. The first candidate we tested was the chromatin remodeling complex, ISW. We investigated the specific roles of ISW in establishing H3K27me₃ and H3K36me₃ in the artificial TetO domain. We generated a new strain (*his-3::tetO tetR-GFP-SUZ12; Δisw*) where we deleted *isw* in the *his-3::tetO tetR-GFP-SUZ12* strain background, and asked if ISW impacts the establishment of H3K27me₃ and H3K36me₃ at the *TetO* array. ChIP-seq results revealed that ISW is not necessary for the establishment of H3K27me₃ (Figure 3.4B: H3K27me₃ track (green)). H3K27me₃ patterns at the TetO array were similar between our *his-3::tetO tetR-GFP-*

SUZ12 and *his-3::tetO tetR-GFP-SUZ12; Δisw* strains. It was reported that in *N. crassa* and other filamentous fungi, ASH1-dependent H3K36 methylation colocalizes with H3K27me_{2/3} (Bicocca et al., 2018). The *isw* mutant in *Neurospora* showed a drastic reduction of H3K36me₃ at PRC2-marked genes (Kamei et al., 2021). Next, we aimed to determine whether ISW is essential for the deposition of H3K36me₃ at the artificial domain. Our results indicated that ISW is indeed necessary for H3K36me₃ deposition at the artificial tetO domain. (Figure 3.4B: H3K36me₃ track (orange)). The *his-3::tetO tetR-GFP-SUZ12; Δisw* lacked H3K36me₃ at the artificial tetO-array compared to the *his-3::tetO tetR-GFP-SUZ12* strain where H3K36me₃ is deposited in the same regions where we see H3K27me₃ at the array. These findings highlight a new function of ISW in regulating PRC2, specifically its role in the establishment of H3K36me₃ at PRC2-targeted domains.

ISW impacts the maintenance of H3K27me₃

ISW had no impact on the establishment of H3K27me₃ at the tetO array. Next, we wanted to know if it was important for maintenance. To test this, we carried out a similar experiment where we grew our *his-3::tetO tetR-GFP-SUZ12* and *his-3::tetO tetR-GFP-SUZ12; Δisw* strains in the absence and presence of tetracycline. We already knew that in the presence of tetracycline, the H3K27me₃ domain is weakly maintained. We wanted to know if H3K27me₃ will be lost faster in the *isw* mutant background (*his-3::tetO tetR-GFP-SUZ12; Δisw*) compared to the wild-type strain (*his-3::tetO tetR-GFP-SUZ12*). We grew our *his-3::tetO tetR-GFP-SUZ12* and *his-3::tetO tetR-GFP-SUZ12; Δisw* strains in liquid Vogel's media and liquid Vogel's media supplemented with tetracycline for 12, 24, 30, and 36 hours (Figure 3.5A). We then performed ChIP-seq to look at H3K27me₃ levels at the tetO array. After the addition of tetracycline, we

noticed that in the absence of ISW, H3K27me3 was lost faster at the artificial tetO array compared to the wild-type. In the wild-type strain (*his-3::tetO tetR-GFP-SUZ12*), H3K27me3 at the tetO-array is detectable for at least 36 hours after the addition of tetracycline (Figure 3.5A). But by 24 hours in the Δisw strain (*his-3::tetO tetR-GFP-SUZ12; \Delta isw*), H3K27me3 is undetectable at the tetO array (Figure 3.5B). These results provide evidence that the ATP-Utilizing Chromatin Assembly and Remodeling Factor (ISW) plays a role in maintaining silent H3K27me domains in *Neurospora crassa*.

Discussion

Our findings showed that an H3K27me3 domain can be initiated by tethering PRC2 to the DNA and that this is sufficient to recruit H3K36 methylation machinery. Moreover, H3K27me3-directed H3K36me3 is dependent on the activity of ISW.. These results suggest the presence of a positive feedback loop between H3K27me3 and H3K36me3, where PRC2 and ASH1 are working together to reinforce silent heterochromatin domains. In mammals, the maintenance of PcG-repressed chromatin depends on the positive feedback loop between PRC1 and PRC2 (Chujo & Scott, 2014a; Dorafshan et al., 2017; Margueron & Reinberg, 2011a; Merini & Calonje, 2015a). Recent studies from mammals and *Drosophila* showed that PRC1 and PRC2 recruit each other, creating a positive feedback loop that results in a stable heterochromatin domain (Blackledge & Klose, 2021; Kahn et al., 2016; Kalb et al., 2014b; Ohtomo et al., 2023; Tamburri et al., 2020). This positive feedback loop between PRC1 and PRC2 ensures that PRC2-marked genes are silenced and maintained over multiple generations. Our results suggest that something similar might be happening between PRC2 and ASH1-mediated H3K36me3. The crosstalk between H3K27me3 and H3K36me3 has been reported in *Neurospora* and other fungi. In the fungal pathogen *Magnaporthe oryzae*, H3K27me3 predominantly marked transposable

elements (TEs). In *Magnaporthe*, loss of H3K27me3 was associated with loss of H3K36me3 from TEs and other gene domains, suggesting a positive interaction between H3K27me3 and H3K36me3 (Wu et al., 2022b; M. Xu, Sun, et al., 2024; W. Zhang et al., 2021). It was reported in *Neurospora* that ASH1-mediated H3K36me3 colocalizes with H3K27me3 (Bicocca et al., 2018). Bicocca et al. concluded that H3K36me2/3 was independent of H3K27me3 and that H3K36me2/3 was upstream of H3K27me3. Our results show that H3K27me3 can recruit H3K36me3 machinery. These results support a new model where H3K27me3 and H3K36me3 work together in a positive feedback loop. H3K36me3 and H3K27me3 can recruit each other to enhance the silencing of PRC2-marked genes.

We also reported that the establishment of H3K36me3 at these domains is dependent on the chromatin remodeling activity of ISW (Barisic et al., 2019; Clapier et al., 2017). The activity of ISWI has been linked to the repression of PRC2-targeted gene domains (Scacchetti et al., 2018). In *Neurospora*, ISW is required for the repression of PRC2-targeted genes and the establishment of normal H3K27 and H3K36 methylation patterns (Kamei et al., 2021; Wiles et al., 2022a). ASH1-dependent H3K36me3 was lost from PRC2-marked genes in the absence of ISW. These results suggested that ISW regulates the activities of both PRC2 and ASH1. We reported that H3K27me3 can be targeted to an artificial domain in the absence of ISW, but H3K36me3 establishment requires ISW. These data suggest that ISW influences PRC2 indirectly by interacting with ASH1 and H3K36me3.

We also found that ISW is required for the maintenance and inheritance of H3K27me3. We already knew that ISW performs a general role in the assembly or maintenance of repressed chromatin states, but the results from this study expand on the specific role that ISW performs. ISWI uses its ATPase domain to space nucleosomes, and this nucleosome remodeling activity is

important for PRC2 recruitment to chromatin (Kamei et al., 2021). Nucleosomes were evenly spaced within PRC2 targeted domains in wild-type *Neurospora* but not in the *isw* mutant (Kamei et al., 2021). Our results show that ISW is critical for the maintenance of H3K27me3. We propose a mechanism where ISW regulates H3K27me3 maintenance by helping create an optimal environment for ASH1 to interact with chromatin via nucleosome spacing. Once this optimal environment is established, ASH1 and PRC2 interact with each other to create a positive feedback loop that results in a stable heterochromatin domain.

In conclusion, our findings illuminate the intricate mechanisms underlying the assembly and maintenance of histone methylation regulation. The tetO/R system opens new avenues of research into the mechanisms underlying the establishment and inheritance of heterochromatic silent domains. The sequence-independent recruitment of PRC2 to an artificial domain, the role of H3K36me3 in conjunction with PRC2, and the essential function of ISWI in regulating the establishment of H3K36me3 and the maintenance of H3K27me3 represent significant contributions to the field of epigenetics. The insights provided by this reporter strain will pave the way for future research aimed at unraveling the complexities of chromatin regulation and its implications for development and disease.

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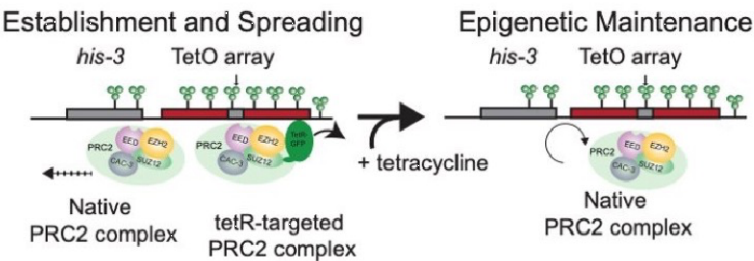
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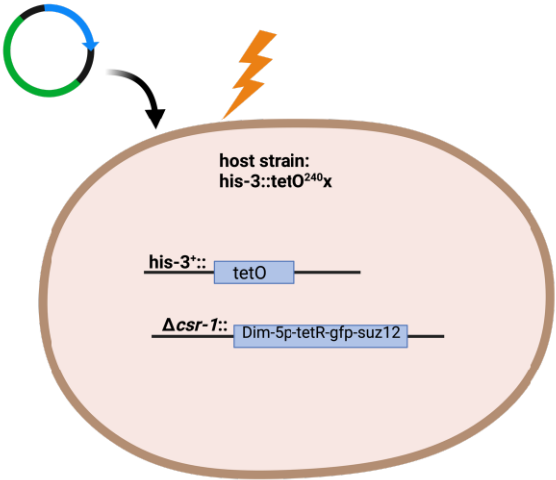
Figures

A

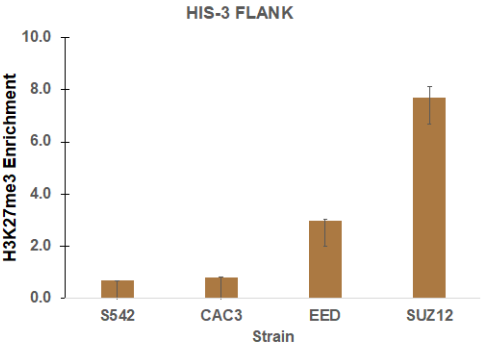


B

[Mini-map from genious]



C



D

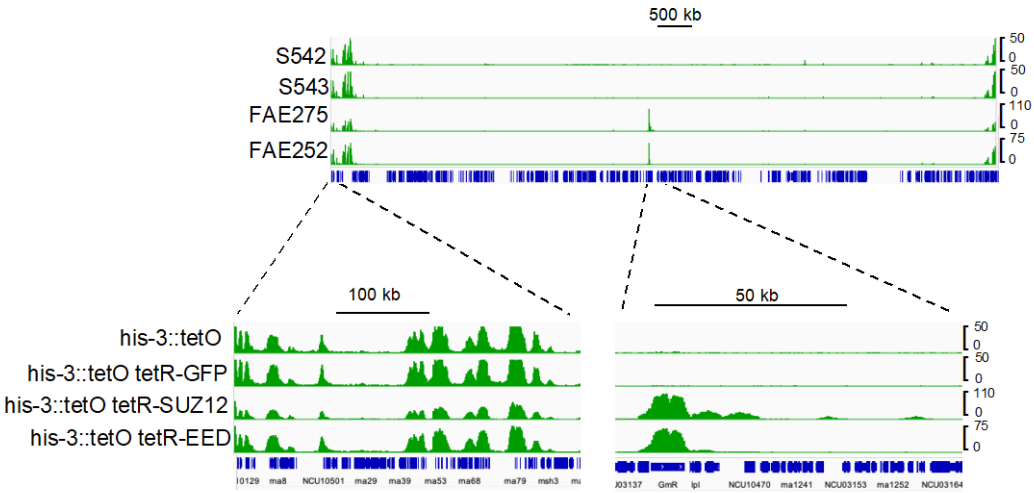


Figure 3.1: Targeting of PRC2 is sufficient to nucleate an artificial H3K27me3. (A)

Schematic cartoon of TetR-GFP-PRC2 at the *csr-1::240XtetO* locus. Tetracycline (tet) promotes the release of TetR-targeted-PRC2 complex from *tetO* sites allowing us to measure establishment and maintenance independently. **(B)** Schematic diagram of cell cartoon with *tetO* at *his-3* and PRC2-fusion at *csr-1* locus. **(C)** ChIP-qPCR measures H3K27me3 at a *his-3* flank region in S542 (*tet-O* only strain), *tetR-GFP-SUZ12*, *tetR-GFP-EED* and *tetR-GFP-CAC-3*. **(D)** The genome browser tracks show the distribution of H3K27me3 across Linkage Group I, which contains the *his-3* locus in S542 (*his-3::tetO^{240x}*), S543 (*his-3::tetO tetR-gfp*), FAE275 (*his-3::tetO tetR-GFP-suz12*) and FAE257 (*his-3::tetO tetR-GFP-eed*). H3K27me3 enrichment was determined by ChIP-seq. The scale bar corresponds to 500 kilobase pairs (kb). The left panel is a magnified genome browser view of a native PRC2 methylated domain with a scale bar of 100 kb. The right panel is a magnified genome browser view of the insertion site of the 240X*tetO-his-3* at the *his-3* locus of chromosome 1 with a scale bar of 50 kb.

A

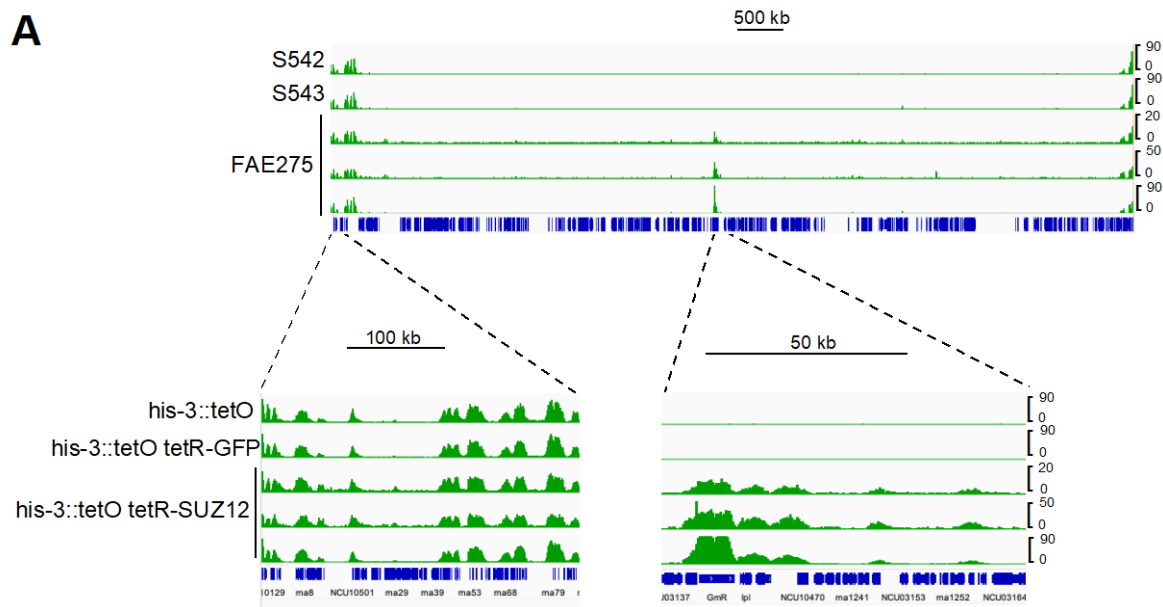


Figure 3.2: Spreading is progressive over time. Genome browser image illustrates H3K27me2/3 enrichment on Linkage Group I for S542 (tet-O only strain), S543 (*his-3::tetO tetR-GFP*), and various passaged strains of FAE275 (*his-3::tetO tetR-GFP-SUZ12*) with a scale bar of 500kb. Two segments of Linkage Group I are displayed at a higher resolution to visualize H3K27me2/3 enrichment. The left panel displays a native PRC2 methylated domain at 100 kb, and the right panel shows the insertion site of the 240XtetO-*his-3* at the *his-3* locus.

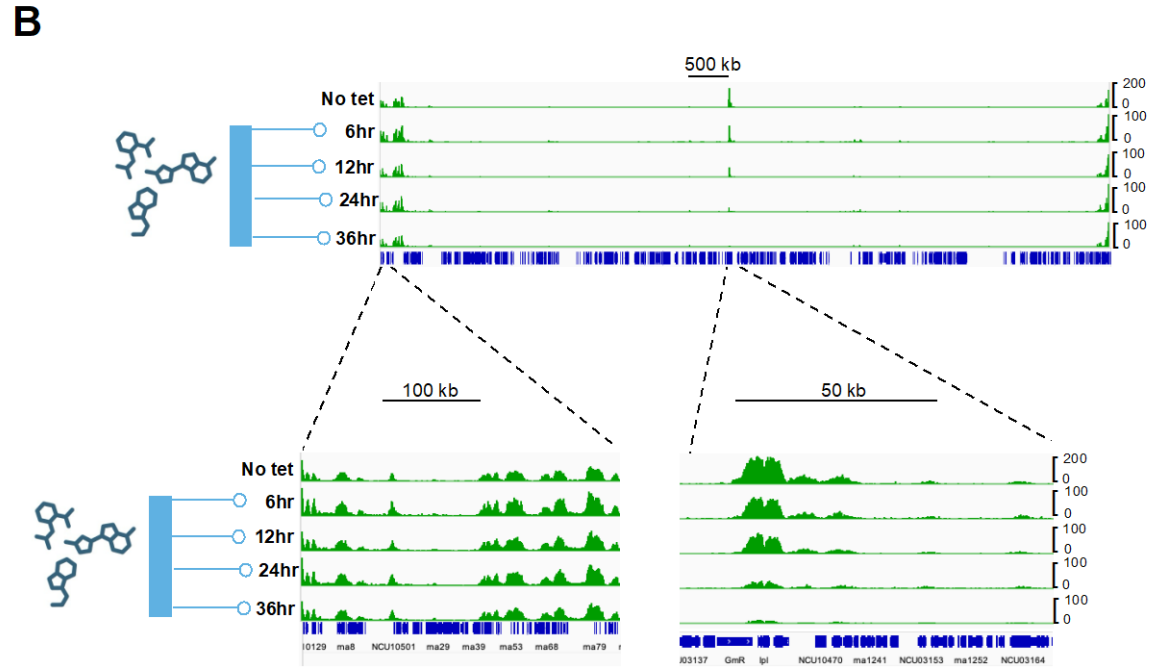
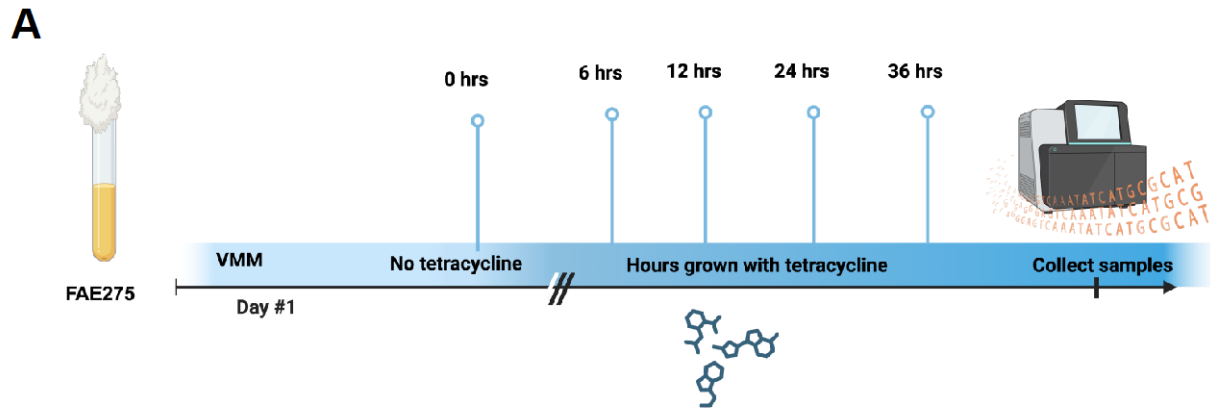


Figure 3.3: H3K27me3 is lost following tetracycline addition. (A) Schematic of the experimental protocol used to measure H3K27me3 following the addition of tetracycline. A slant of FAE275 (*tetR-GFP-SUZ12*) was inoculated in VMM (Vogel's minimal media) grown with no tetracycline for 36 hrs and with tetracycline for 6 hours, 12 hours, 24 hours, and 36 hours with a scale bar of 500 kb. (B) Browser shot showing H3K27me3 ChIP-seq data for FAE275 grown with tetracycline for 6, 12, 24, and 36 hours. The left panel displays a native PRC2 methylated domain at 100 kb, and the right panel shows the insertion site of the 240XtetO-*his-3* at the *his-3* locus.

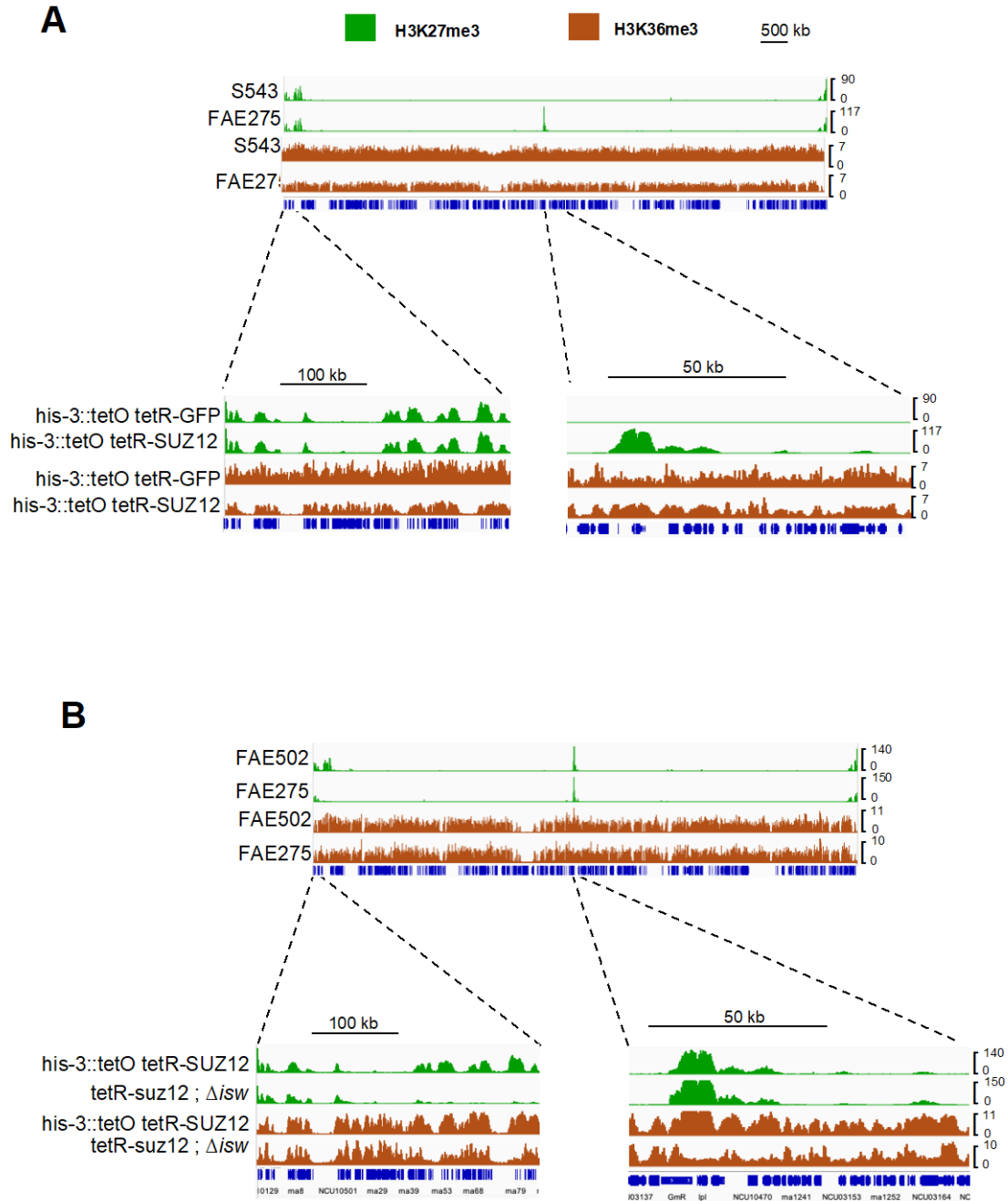


Figure 3.4: H3K27me3 recruits H3K36me3, and this depends on ISW. (A) Genome browser image of ChIP-seq for H3K27me3 (green) and H3K36me3 (orange) in (S543) *his-3::tetO tetR-GFP* and (FAE275) *his-3::tetO tetR-GFP-SUZ12* in Linkage Group I. The left panel displays a native PRC2-methylated domain at 100 kb, and the right panel shows the insertion site of the 240XtetO-*his-3* at the *his-3* locus. **(B)** Genome browser of H3K27me3 (green) and H3K36me3 (orange) in (FAE275) and (FAE502) *tetR-GFP-SUZ12; Δisw*. The left panel shows a native PRC2 methylated domain at 100 kb, and the right panel shows the insertion site of the 240XtetO-*his-3* at the *his-3* locus.

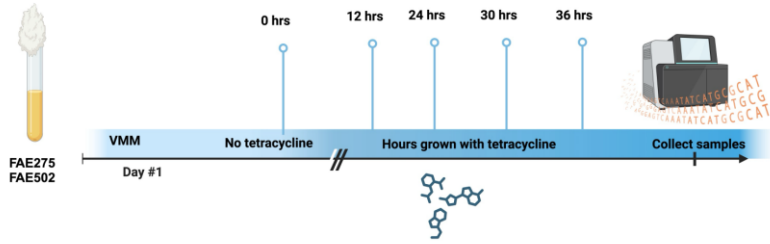
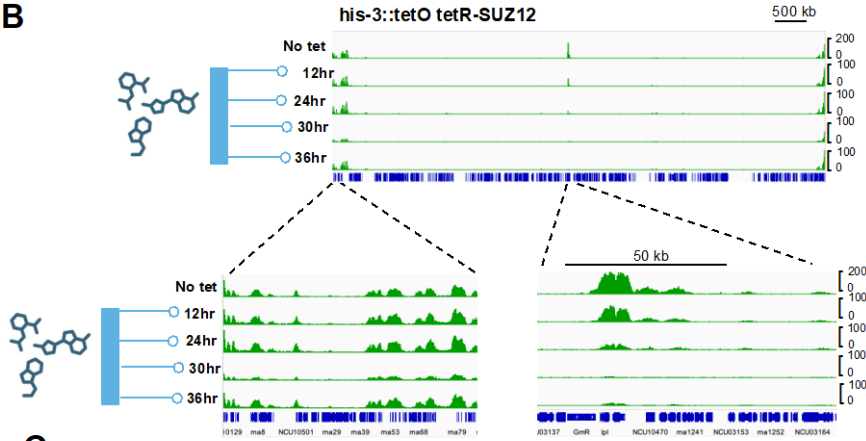
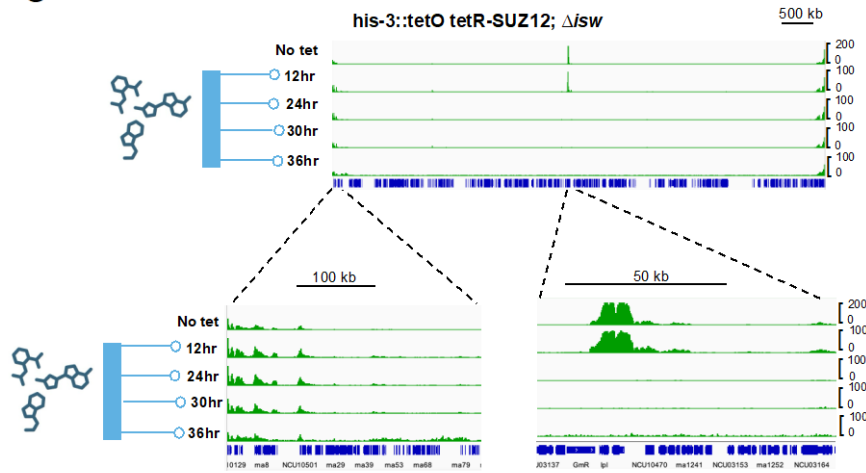
A**B****C**

Figure 3.5: ISW is required for the maintenance of H3K27me3 at targeted domains. (A) Schematic of the experimental protocol used to measure H3K27me3 following the addition of tetracycline. A slant of FAE275 (*tetR-GFP-SUZ12*) and FAE502 (*his-3::tetO tetR-GFP-SUZ12; Δisw*) was grown in VMM (Vogel's minimal media) with no tetracycline for 36 hrs and with tetracycline for 12, 24, 30, and 36 hours. The scale bar of 500 kb. **(B)** Browser shot showing H3K27me3 ChIP-seq data for FAE275 grown with tetracycline for 12, 24, 30, and 36 hours. **(C)** Browser shot showing H3K27me3 ChIP-seq data for FAE502 grown with tetracycline for 12, 24, 30, and 36 hours. The left panel displays a native PRC2 methylated domain at 100 kb, and the right panel shows the insertion site of the 240XtetO-*his-3* at the *his-3* locus.

Establishment and Spreading

Epigenetic Maintenance

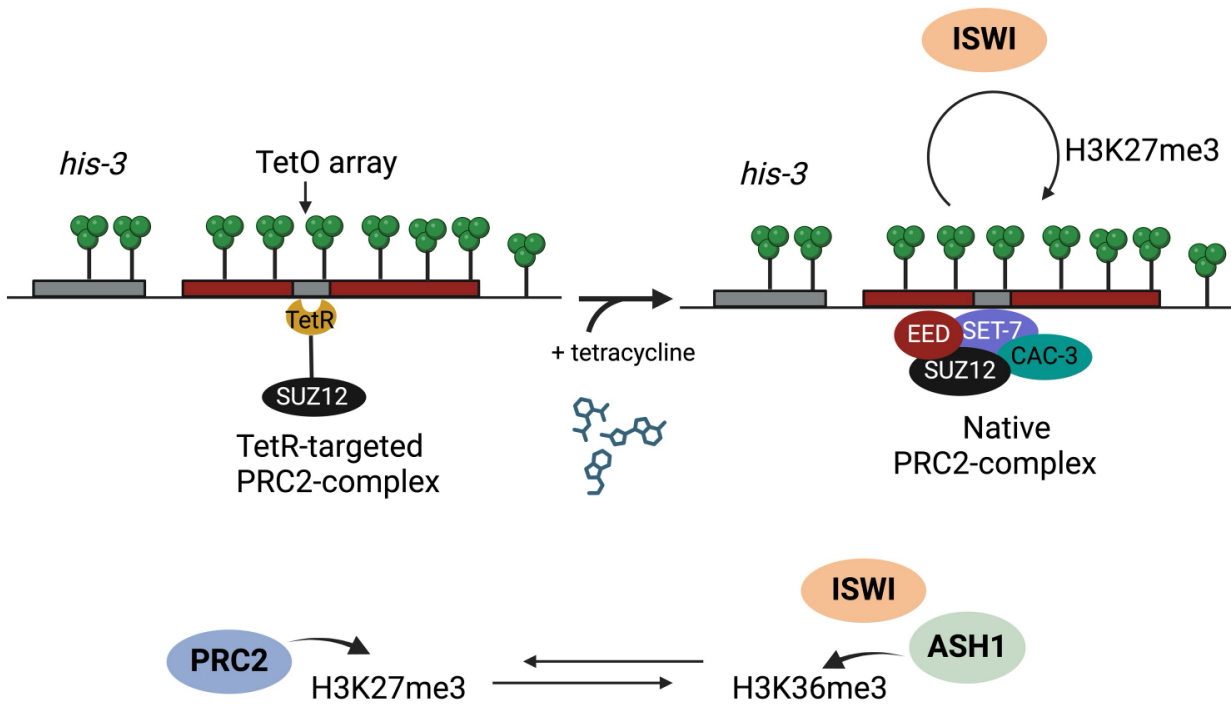
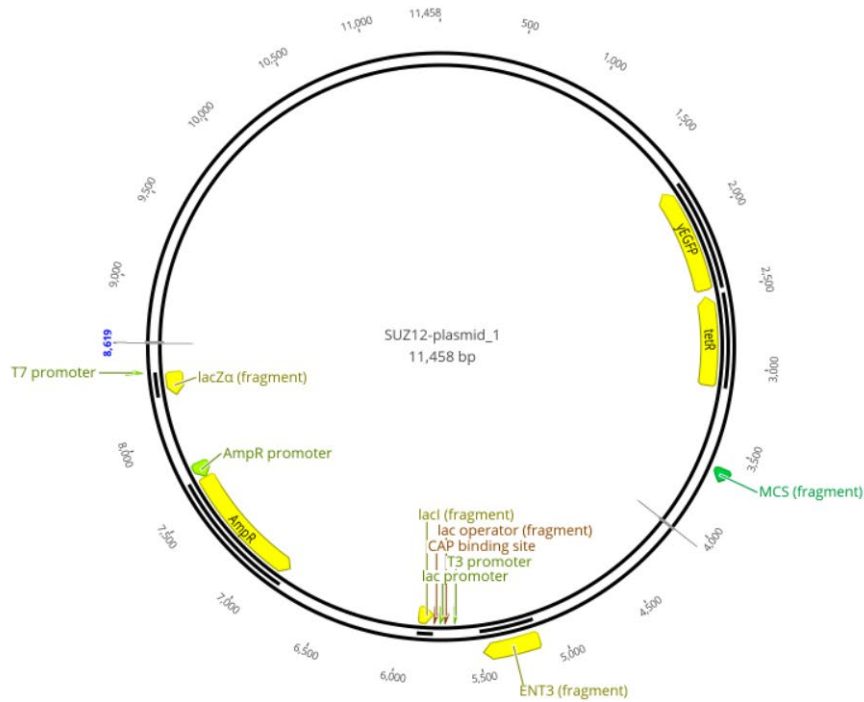


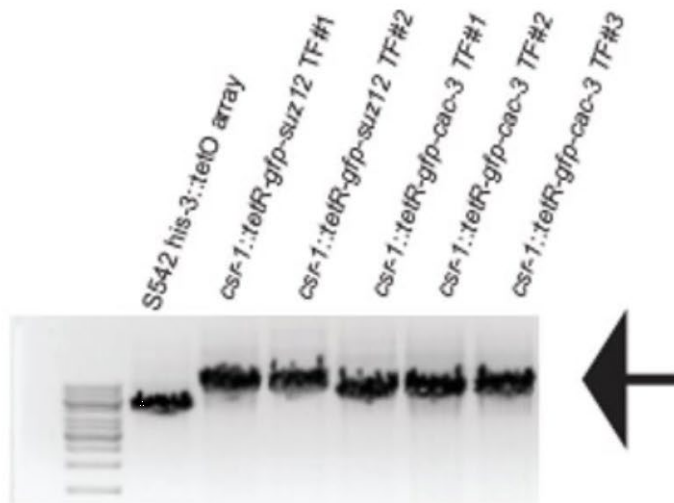
Figure 3.6: A model summarizing our findings. We showed that SUZ12 is sufficient to establish an ectopic domain of H3K27me3. The maintenance of this domain is dependent on ISW. We also showed that H3K27me3 can recruit H3K36me3, and this is dependent on ISW. PRC2 and ASH1 work together in a positive loop to maintain silent heterochromatin states in *Neurospora crassa*.

Supplementary Figures

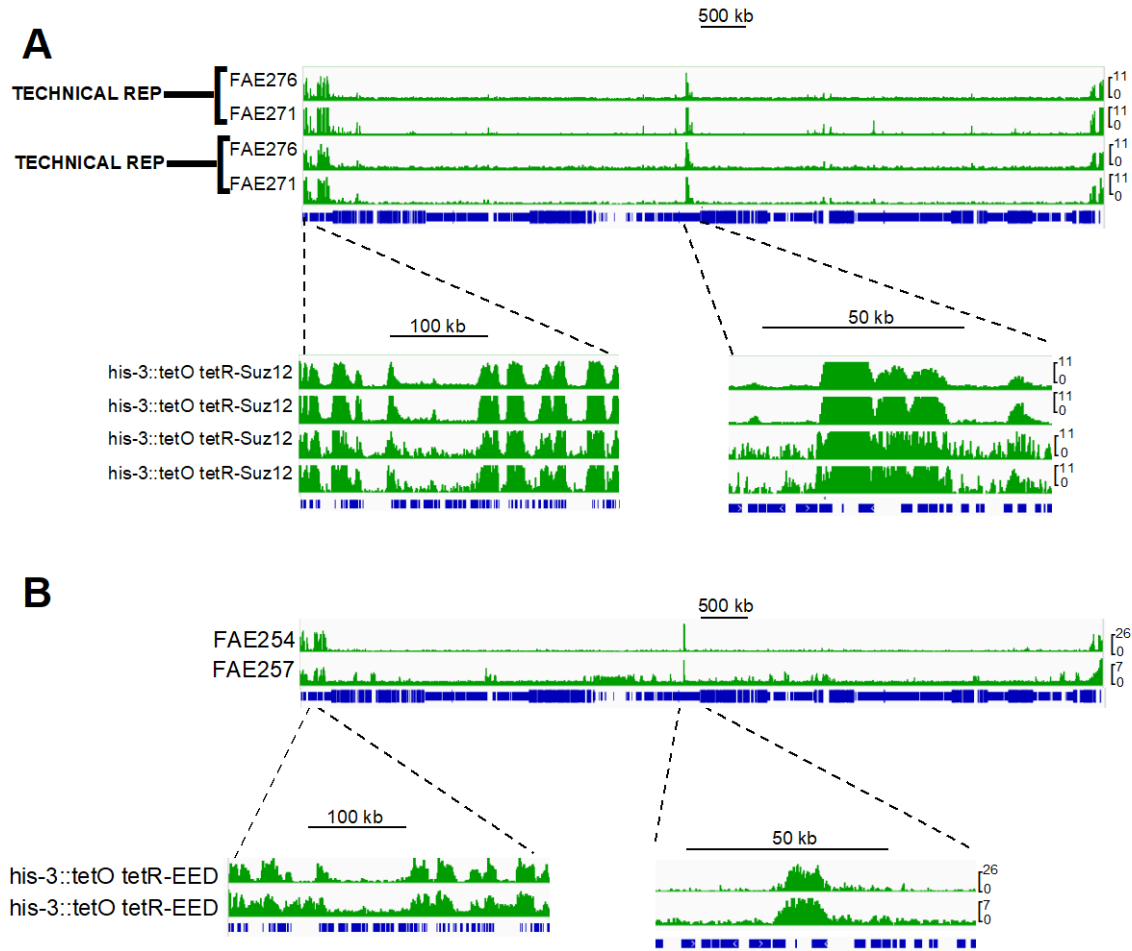
A



B



Supplementary Figure 3.1: Validation of tetR-PRC2 plasmids. **(A)** Plasmidsaurus map confirming that the constructed plasmids were correct. **(B)** PCR results showed that the primary transformants had the correct size plasmids inserted into the *csr-1* locus.



Supplementary Figure 3.2: Technical and biological replicates confirming that EED and SUZ12 are sufficient to target H3K27me3 at the tet-O array. (A) Technical and biological replicates of ChIP-seq data showing H3K27me3 enrichment for the *his-3::tetO tetR-GFP-SUZ12* strains. **(B)** Technical and biological replicates of ChIP-seq data showing H3K27me3 enrichment for the *his-3::tetO tetR-GFP-EED* strains.

Tables

Table 3.1: Primers used in this study

Name	Sequence (5'-----3')
ISWI KO FP	TGGTCTAGCTGCTAAAGAGG
ISWI KO RP	TTATGTACGAGGTCGAGAGG
hisflank_1fwd	TAACGTCAAGACACATAGCTACCT
hisflank_rev	TCATGTGCTCAAGACGGATGCTGAC
hisflank3_rev	ACACAAATGGAAAACGGGACTC
hisflankfwd_3	CTACCCCGTCAAATCTATACATATC
Histone H4 5' FP1	GTGATGCCGTCACCTACAC
Histone H4 5' RP1	ACGGCGAGACATTTAACCA
Histone H4 5' FP2	CACACTTCACTTGGTCGCA
Histone H4 5' RP2	ATGACCCCTTGATGTGATGTA
Csr-1 genotype fwd	GATGCCAAGAAATCCCGGGA
Csr-1 genotype rev	TCGCCAACACATTGAATGCT
SUZ12rev	GGGTTGTGCGAGGTGTGG
SUZ12fwd	TCCGTCTTTTGGGTAGCTAGAT
EED-geno rev	GAGAGGGGAGGAGGAAGAGG
EED-geno fwd	ATGCAGTGAAGAAGGGTGGG
csr1 3p RP w overhang NEW	AATTAATACGACTCACTATAGGGAGGCCCTGGTTTACTGAGGGC
csr1 5 FP overhang	CACTATAGAACTCGAGCAGCTGAAGCAACACCTCCGTCGCCATAAAC TCC
pBM61_inversePrimer2	TCCCTATAGTGAGTCGTATTA
PBM61_inversePrimer1	GCTTCAGCTGCTCGAGTTCT

Table 3.2: Strains used in this study

Genotype	Strain #	Additional information	Reference
<i>Wildtype</i>	S2		
<i>his-3+::tetO ; mus-52::bar+</i>	S542	T75.12h (S542) transformed with pEAG249G (linearized with <i>AndI</i>)	
<i>his-3+::tetO; csr-1::p[TrpC]::TetR::eGFP ; mus-52::bar+</i>	S543	C146.1 (G&K Na Gen 2017) transformed with pEAG62A	
<i>his-3::tetO tetR-Suz12</i>	FAE275		This study
<i>his-3::tetO tetR-Suz12</i>	FAE271		This study
<i>his-3::tetO tetR-Suz12</i>	FAE276		This study
<i>his-3::tetO tetR-EED</i>	FAE257		This study
<i>his-3::tetO tetR-EED</i>	FAE254		This study
<i>tetR-suz12 ; Δisw</i>	FAE502		This study
<i>tetR-suz12 ; Δisw</i>	FAE503		This study

CHAPTER 4

DISCUSSION

The work presented in this dissertation represents significant contributions to our understanding of the function and regulation of facultative heterochromatin in filamentous fungi. By focusing on *Neurospora crassa* as a model organism, this research provides novel insights into the intricate mechanisms that govern the assembly, maintenance, and regulation of facultative heterochromatin. Facultative heterochromatin plays a crucial role in cellular differentiation and development, enabling cells to respond to environmental signals and developmental cues by modulating gene expression. Through a series of experimental approaches, this dissertation elucidates the regulatory pathways involving key players such as the Polycomb Repressive Complex 2 (PRC2) and Histone Deacetylase 1 (HDA-1), revealing how these complexes interact to maintain chromatin integrity and transcriptional silencing. Additionally, these findings highlight the consequences of perturbations in these regulatory mechanisms, such as the global redistribution of H3K27 methylation observed upon *hda-1* deletion, which underscores the delicate balance required for proper chromatin function. By establishing a reporter system to distinguish between the establishment and maintenance of H3K27me3 marks, this research also advances our methodological toolkit for studying chromatin regulation, providing a framework for future investigations. Overall, this dissertation not only enhances our comprehension of facultative heterochromatin in filamentous fungi but also

contributes to the broader field of epigenetics by offering new perspectives on how chromatin regulation can influence gene expression and cellular identity in eukaryotic organisms.

Histone Deacetylase 1 (HDA-1) regulates PRC2 activity

The study of Polycomb repression in *Neurospora crassa* opens several important avenues for future research, particularly regarding the interactions between PRC2 and other epigenetic factors. While much of the existing literature has explored the crosstalk between PRC2 and various epigenetic machinery across different organisms, including plants, animals, and fungi, there remains a critical need to investigate these crosstalk in *N. crassa*. Previous studies have documented cooperative and antagonistic relationships between PRC2 and other epigenetic regulators (Bicocca et al., 2018; Chittock et al., 2017; Cifuentes-Rojas et al., 2014; Pasini et al., 2010; Schmitges et al., 2011; Tie et al., 2009; B. Xu et al., 2015; Yuan et al., 2011, 2012).

Our genetic screen aimed to identify genes essential for Polycomb repression, and this has led to some compelling findings, particularly regarding the role of Histone Deacetylase 1 (HDA-1). Our results indicate that HDA-1 is crucial for the repression of PRC2-targeted genes. In the absence of HDA-1, we observed a significant loss of H3K27me_{2/3} from typical PRC2-targeted domains, accompanied by an aberrant accumulation of these marks at constitutive heterochromatin regions characterized by H3K9me₃. This redistribution is particularly noteworthy because it highlights the delicate balance maintained by histone modifications and suggests a potential mechanism for transcriptional dysregulation. Additionally, we identified chromodomain protein 2 (CDP-2), another mutant in the *Neurospora* histone deacetylase complex (HCHC), as important for the proper localization of the H3K27me_{2/3} methylation mark. The functional relationship between CDP-2 and PRC2 in *N. crassa* warrants further

exploration, particularly in understanding how these interactions contribute to the establishment and maintenance of facultative heterochromatin.

Our findings also suggest that the loss of HDA-1 leads to epigenome instability, evidenced by changes in other histone modifications such as H3K36me3 and pan-acetylation. This observation raises intriguing questions about the broader implications of histone deacetylase activity in maintaining genomic integrity and transcriptional fidelity. While the relationship between histone deacetylases and PRC2 has been minimally studied, there are notable examples in the literature that can guide future investigations. For instance, in embryonic stem cells, the NuRD complex, which possesses deacetylation activity, was shown to recruit PRC2 to target promoters, thereby silencing genes that are critical for development (Layman et al., 2013; Reynolds et al., 2011). Moreover, a synergistic relationship between PRC2 and HDAC1 complexes in mediating transcriptional repression has been observed in murine erythroleukemia (Gregoricchio et al., 2022). Additionally, in the rice fungal pathogen *Magnaporthe oryzae*, the Sin3 histone deacetylase complex was found to be essential for the proper distribution of H3K27me3 occupancy and associated transcriptional silencing (Lin et al., 2022).

This dissertation adds to the collective understanding of how histone deacetylases regulate PRC2 function in fungi. By elucidating the mechanisms by which HDA-1 influences PRC2-targeted gene repression and histone modification regulation, this work not only advances our knowledge of Polycomb repression in *Neurospora* but also provides a foundation for future studies aimed at exploring the intricate interplay between various epigenetic regulators in fungal biology.

Uncoupling H3K27me3 establishment and maintenance in the model fungus *Neurospora crassa*.

To fully comprehend the regulation of heterochromatin, it is crucial to differentiate between the processes of assembly and maintenance of heterochromatin "states," as these phenomena are distinct yet interrelated. Recognizing this distinction is fundamental for understanding how epigenetic modifications influence gene regulation over time. In pursuit of this understanding, we developed an inducible system specifically designed for the formation of facultative heterochromatin. By fusing subunits of the Polycomb Repressive Complex 2 (PRC2)—namely EED, SUZ12, and CAC3—to the bacterial TetR protein, we leveraged the TetR DNA binding domain to facilitate targeted recruitment of these proteins to a specific genomic locus containing its cognate binding sequence, the TetO array. In this study, we showed that artificial targeting of PRC2 establishes an ectopic domain of H3K27me₃. This approach allows us to manipulate the assembly of facultative heterochromatin in a controlled manner, enabling a more precise investigation of the underlying mechanisms.

The utility of this system is further enhanced by its design: the recruitment activity of the TetR-fused PRC2 components can be rapidly abrogated by the addition of tetracycline, effectively creating a Tet-Off system. Our results confirmed that H3K27me₃ at the artificial domain is lost following tetracycline addition. This allows for the dynamic regulation of heterochromatin formation, providing a powerful tool for studying how the establishment of H3K27me₃ marks occurs in real-time. By using this system, we can address several unresolved questions related to chromatin regulation. For instance, we can investigate how quickly and efficiently the PRC2 complex assembles on the targeted locus and what factors influence its recruitment. Additionally, we can assess how the removal of PRC2 affects the stability of the H3K27me₃ mark and whether this leads to changes in gene expression.

This research not only contributes to our understanding of facultative heterochromatin regulation but also opens avenues for exploring the broader implications of chromatin regulation in various biological contexts. Future studies utilizing this inducible system could provide deeper insights into the roles of different PRC2 subunits, the impact of other epigenetic factors on heterochromatin formation, and the potential consequences of disrupted chromatin states on cellular function and development. We have begun to explore the impact of other epigenetic factors on facultative heterochromatin formation. Work from this study showed that H3K27me3 recruits H3K36me3, and this depends on the well-characterized chromatin remodeling enzyme ISWI (Kamei et al., 2021; Wiles et al., 2022a). Our lab and others have identified several chromatin-associated proteins (H2A.Z, PAS, CAC-1,-2, -3, EPR-1, RPD3L, HDA-1, and ASH-1) that are required for normal H3K27me3 patterns (Basenko et al., 2015; Bicocca et al., 2018; Courtney et al., 2020; Jamieson et al., 2018; McNaught et al., 2020; Mumford et al., 2024; Wiles et al., 2020). Future studies can take advantage of this system to identify mutants that are defective in heterochromatic establishment and maintenance.

In summary, our study lays the groundwork for a nuanced exploration of heterochromatin regulation, emphasizing the importance of assembly and maintenance as distinct yet interconnected processes. By establishing a robust platform for investigating these phenomena, we hope to shed light on the intricate regulatory networks governing gene expression and chromatin structure in eukaryotic cells.

Boundaries regulating H3K27me3 spreading in *Neurospora crassa*

The establishment of the reporter system in this study marks a significant advancement in our ability to investigate the mechanisms governing the spreading of H3K27me3 within *Neurospora*. By generating a novel reporter strain that incorporates a mCherry fluorescence tag

fused to six tetO array repeats, we have provided a versatile tool for future research that has been highlighted in Appendix A. The confirmation of mCherry expression via microscopy reinforces the viability of this system as a reliable indicator of chromatin modifications.

Our introduction of the tetR-GFP-SUZ12 construct into the reporter strain demonstrated that PRC2 effectively represses mCherry expression, a finding that was further validated through ChIP-seq analysis. This repression highlights the critical role of PRC2 in modulating chromatin states and suggests that our system can be employed to unravel the complexities of chromatin regulation. Notably, we also explored the potential for controlling the tetOFF system using tetracycline, which resulted in the loss of H3K27me3. This feature of the reporter system not only underscores its dynamic capabilities but also opens avenues for real-time studies of chromatin behavior. The random insertion of the 6x tetO array repeats into the *Neurospora* genome lays a foundation for further explorations into the spreading mechanisms of H3K27me3.

Current literature has extensively documented the spreading of H3K9me3 in organisms such as yeast and higher eukaryotes (Al-Sady et al., 2013; Grewal, 2023; Grewal & Jia, 2007; Hall et al., 2002, 2002; Noma K et al., 2001; Rea et al., 2000; Stewart et al., 2005; J. Wang et al., 2014; K. Zhang et al., 2008). For instance, in *Saccharomyces cerevisiae*, the silent chromatin at telomeres and the mating-type loci are intricately linked to the SIR proteins, which mediate the telomere position effect (Kueng et al., 2013; Rusche et al., 2003; Schotta et al., 2003; Talbert & Henikoff, 2006). Insights from *Drosophila* suggest that PRC2 is predominantly localized at Polycomb response elements (PREs), yet H3K27me3 marks can extend across large chromosomal domains (Schwartz et al., 2006). Various hypotheses regarding the mechanism of H3K27me3 spreading, including local diffusion of PRC2 and the formation of chromosome loops, warrant further investigation (Schwartz & Pirrotta, 2007; Talbert & Henikoff, 2006). In

Neurospora, while we recognize that H3K27me3 covers large multi-gene domains (Aramayo & Selker, 2013; Jamieson et al., 2013b, 2018), there remain critical questions regarding how H3K27me3 deposited by PRC2 spreads beyond its nucleation site. Future research utilizing this reporter strain should prioritize identifying specific boundaries that regulate H3K27me3 spreading.

We plan to generate additional reporter strains that incorporate a mCherry fluorescence tag fused to six tetO array repeats strategically inserted into various regions of the *Neurospora* genome. This will allow for a comprehensive assessment of how different genomic contexts influence the spreading and maintenance of chromatin modification. Next, we will introduce the tetR-GFP-SUZ12 construct into these reporter strains and perform chromatin immunoprecipitation followed by sequencing (ChIP-seq). This analysis will enable the comparison of H3K27me3 occupancy across different regions, helping to determine whether certain genomic locations facilitate spreading more effectively than others. In conjunction with ChIP-seq, we will employ fluorescence microscopy to examine the expression levels of the mCherry fluorescence tag. By evaluating whether different genomic regions promote mCherry expression variably, we aim to gain insights into whether the spreading or assembly of PRC2 is region-specific or random.

Moreover, the implications of H3K27me3 spreading are profound, as it can disrupt normal gene expression patterns, leading to detrimental effects on organismal fitness and health. This is particularly evident in human diseases associated with uncontrolled heterochromatin spreading (Kleinjan & Lettice, 2008). In *Neurospora*, evidence suggests that disruptions in heterochromatin boundary formation can result in growth defects due to the unchecked spreading of silenced chromatin and DNA methylation (Honda et al., 2010). Thus, future investigations

should harness this reporter strain to address these unresolved questions regarding the mechanisms and factors influencing H3K27me3 spreading in *Neurospora crassa*.

In conclusion, this study not only provides a foundational tool for examining H3K27me3 regulation but also emphasizes the importance of understanding chromatin regulation within a broader biological context. As we continue to dissect these mechanisms, we pave the way for future discoveries that could illuminate the complexities of gene regulation in various organisms.

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APPENDIX A

ECTOPIC TARGETING OF H3K27ME3 IS SUFFICIENT FOR GENE REPRESSION

Abstract

This study aims to investigate the mechanisms regulating the spreading of H3K27me3 domains in *Neurospora* through a novel genetic system. We first confirmed that the addition of the PRC2 complex is sufficient for repressing gene expression, as demonstrated by reduced mCherry fluorescence in strains containing 6x tetO repeats. Our ChIP-seq results revealed that, in the absence of PRC2, there was no H3K27me3 enrichment at the artificial domain. However, upon introducing PRC2, we observed significant H3K27me3 marks that spread beyond the nucleation site, indicating the complex's role in both establishing and propagating this modification. Additionally, the reporter system will be used to further explore the mechanisms that control the spreading of H3K27me3 and to identify heterochromatin boundaries in *Neurospora*. These findings contribute valuable insights into factors that impact the spread and maintenance of H3K27me3.

Introduction

Eukaryotic DNA is organized into nucleosomes—147 bp of DNA wrapped around an octamer of histone (Kornberg, 1974; Kornberg & Lorch, 1999; Luger et al., 1997). The histones are marked with epigenetic modifications, such as histone modifications, which alter the expressions of genes without changing the underlining DNA sequences (Brickner, 2023; Henikoff & Greally, 2016). Some of these histone modifications are involved in regulating key biological and cellular processes such as development, differentiation, and various disease states (Schuettengruber et al., 2017). These histone post-translational modifications also regulate chromatin structure and function (Presman et al., 2024). Chromatin in the nucleus is partitioned into two primary types – euchromatin and heterochromatin – and different post-translational modifications affect the different types of chromatins (Grewal & Jia, 2007; J. M. Lee et al.,

2023). Euchromatin is marked with active transcriptional modifications and is less compact, making it more accessible to transcription factors. In contrast, heterochromatin is marked with repressive modifications and is more densely packed. Heterochromatin is further divided into constitutive and facultative heterochromatin. Genes in constitutive domains are always silent, while those in facultative heterochromatin domains can switch between being active and inactive based on cellular signals.

Trimethylation of histone H3 at lysine 27 (H3K27me3) marks facultative heterochromatin and is associated with long-term gene silencing (Guo et al., 2021). A group of proteins known as Polycomb Group (PcG) proteins function in regulating facultative heterochromatin in plants, animals, and fungi (Hennig & Derkacheva, 2009b; Kassis et al., 2017b; Z. A. Lewis, 2017). These proteins were first characterized in *Drosophila melanogaster*, where they silence homeotic (Hox) genes, a gene family controlling thoracic and abdominal development (E. B. Lewis, 1978). These proteins ensure the silencing of genes involved in key developmental processes such as X-chromosome inactivation (Wutz, 2011) and genomic imprinting (Terranova et al., 2008). PcG proteins in mammals form two large complexes: the Polycomb Repressive Complexes 1 and 2 (PRC1 and PRC2). H3K27 methylation is catalyzed by PRC2 (Kouznetsova et al., 2019; Presman et al., 2024), and H3K27me3 deposited by PRC2 silences facultative heterochromatin domains.

In *Drosophila*, PRC2 is recruited to targeted domains via DNA segments known as Polycomb Response Elements (PREs). PRC2 uses its catalytic subunit EZH2 to deposit H3K27 methylation at the targeted domain. Another PRC2 subunit, EED, binds the deposited H3K27me3 and helps it spread beyond the nucleation site (Yang & Li, 2020). Gene silencing at these domains is stabilized by PRC1. The H3K27me3 deposited by PRC2 serves as a binding site

for PRC1. PRC1 ubiquitinates histone H2A at lysine 119 (H2AK119ub), leading to long-term gene silencing of heterochromatin domains. PREs have not been identified in mammals, plants, or fungi, suggesting that PRC2 does not require the pre-existence of repressive histone marks to be recruited in these systems. Multiple studies have reported the role of unmethylated CG-rich sequences, such as CpG islands (CGIs), RNA molecules, and other non-PRC2 proteins in mediating PRC2 recruitment in mammals (Li et al., 2017; Lynch et al., 2012; Mathieu et al., 2005).

PRC2 can be targeted in a *de novo* manner, independent of the catalytic activity of the complex (Højfeldt et al., 2018). This was accomplished by targeting PRC2 subunits to an artificial domain, leading to the *de novo* establishment of H3K27me_{2/3} at the target region (Hernández-Romero & Valdes, 2022). These ectopically deposited sites of heterochromatin are maintained over multiple generations (Brickner, 2023; Ragunathan et al., 2015). In *Neurospora*, we showed that targeting the PRC2 subunits EED and SUZ12 is sufficient to establish an ectopic H3K27me₃ domain. We generated a working model for how steady-state H3K27me₃ patterns are generated and maintained in *Neurospora* (Supplementary Figure 1). In this model, the initial establishment of H3K27me₃ is separated from the maintenance of the H3K27me₃ at the silent heterochromatic domain. First, PRC2 is recruited to naive chromatin, where it methylates H3K27. Once the site is nucleated by PRC2, H3K27 methylation spreads along the chromatin fiber using its “read-write” mechanism. Finally, the established H3K27me₃ must be maintained over multiple cell divisions. The goal of this study was to test whether our model for how steady-state H3K27me₃ patterns are generated and maintained in *Neurospora* is correct.

To do so, we took advantage of our simple model system, *Neurospora crassa*. Very little is known about how PRC2 is regulated in *Neurospora* and other fungi. *Neurospora*’s Polycomb

Repressive Complex 2 (PRC2) contains the SET domain protein SET-7, which is responsible for catalyzing H3K27me₃. The *Neurospora* PRC2 complex also includes the histone-binding protein CAC-3, the DNA-binding protein SUZ12, and EED. PREs have not been identified in *Neurospora* or any other fungi. However, cis-acting sequences were shown to recruit PRC2 in *Neurospora*. Targeting of telomere repeat sequences was sufficient to induce a large H3K27me₃ domain (Jamieson et al., 2018). Like plants and animals, PRC2 recruitment in *Neurospora* is mediated by factors such as CAC-3/NPF, PAS, EPR-1, HZA.Z, and the chromatin remodeling complex ISWI (Courtney et al., 2020b; Jamieson et al., 2013a; Kamei et al., 2021; McNaught et al., 2020; Wiles et al., 2020). We generated a system that allows us to separate the establishment of H3K27me₃ from maintenance. We saw that the ectopic establishment of H3K27me₃ was sufficient for gene silencing. Once the H3K27me₃ was nucleated, it spread and silenced neighboring genes. We reported that the ectopically established H3K27me₃ domain was weakly maintained over time. Finally, we proposed an experimental design where we can use this system to uncover the mechanisms regulating the spread of H3K27me₃ beyond the nucleation site.

Materials and Methods

Culture growth conditions

Vegetative cultures were initiated from *N. crassa* macroconidia and cultivated in Vogel's minimal medium with 1.5% sucrose and shaken at 32°C for 18 hours unless stated otherwise in the results. Tetracycline was added to the growth medium at a final concentration of 5 µg/ml where indicated. All strains used in this study are listed in Table S1, and the primers used to build our tetR-GFP-PRC2 strains are listed in Table S2.

6x-12xTetO – mCherry-NLS plasmid construction

We generated two plasmids: one with 6X TetO repeats, and another with 12X TetO repeats to determine the minimal number of TetO sequences required to recruit the tetR binding protein (Supplementary Figure 2). The TetO repeats were positioned between a mCherry reporter and a hygromycin-resistance cassette. A nuclear localization sequence (NLS) was attached to the construct to ensure targeting into the nucleus. Additionally, the plasmid contained a hygromycin-resistance gene, which facilitated the selection of correctly transformed organisms. *E. coli* transformations were performed to generate additional copies of each plasmid. Plasmid DNA was extracted from these cultures using a mini-prep protocol (Catalog # 50-444-186). Upon purification, 5 µg of each plasmid was subjected to digestion with the SphI restriction enzyme to linearize the plasmids for transformation into *Neurospora crassa* strains S20 and S2. The S2 strain, a wild-type strain, was utilized for ectopic insertion of the plasmids into *Neurospora crassa*. For the transformation step, 300 ng of digested 6x and 12x tet-R-mCherry plasmids were combined with 40 µL of the prepared conidial suspension. The mixture was subjected to electroporation using a Biorad electroporator (BTX Electro Cell Manipulation 600, Genetronics) set to 1.5 kV, 600 ohms, and 25 µF, conditions optimized to enhance the uptake of the plasmids into the conidia. After electroporation, the cells were immediately recovered in 960 µL of cold 1M sorbitol. Finally, the transformed conidial suspension was evenly spread on hygromycin B selection plates and incubated in a 32°C incubator for a duration of 3 to 5 days.

Construction of 6xTetO – mCherry tetR-GFP-SUZ12 strain

We introduced the tet-R-GFP-SUZ12 construct into the host strain containing the 6xTetO-mCherry-NLS. This was integrated into the *csr-1* locus of our host strain via transformation as described in (Margolin et al., 1997). 200 µl of transformation solution was

spread on agar medium plates containing Cyclosporin A (Sigma #30024) selection: 5 µg/ml final concentration from 2.5 mg/ml stock (500X) diluted in ethanol. Colonies resistant to Cyclosporin A were isolated and tested by PCR using the *csr-1* genotyping primers to determine whether the *csr-1* gene is replaced and if the transgene tetR-GFP-SUZ12 is integrated into the *csr-1* locus (Supplementary Figure 3).

Microscopy Imaging of mCherry strains

Conidia were suspended in 20 µL of water to create a concentrated suspension, which was subsequently placed onto microscope slides for analysis. Imaging was performed using an ECHO Revolve microscope (model #RVSF1000/Revolve R4) to check for the expression of the mCherry reporter gene within the transformed *Neurospora* strains.

Chromatin immunoprecipitation (ChIP-seq)

ChIP-seq was performed as described with the following antibodies H3K27me3 (Abcam Cat# ab6002) (Ferraro & Lewis, 2018). Libraries were created and analyzed as described (Ferraro & Lewis, 2018). The libraries were submitted for sequencing at the Georgia Genomics Facility at the University of Georgia on a NextSeq 500 instrument.

Results

An experimental system to study establishment, maintenance, and spreading in *Neurospora crassa*

De novo H3K27me3 can be established at a targeted locus (Hernández-Romero & Valdes, 2022; Højfeldt et al., 2018). We had previously shown that targeting TetR protein fused to the PRC2 subunits EED and SUZ12 was sufficient to establish an ectopic H3K27me3 domain

at a TetO-array containing 240 copies of the TetR binding site. However, we could not determine if the ectopically established H3K27me3 was sufficient for gene silencing. To address if an ectopically targeted H3K27me3 was sufficient for gene silencing in *Neurospora*, we modified our TetO/TetR system. We added an mCherry reporter gene next to the 6X-Tet O array to test if targeting of H3K27me3 could lead to the silencing of mCherry expression (Supplementary Figure 4). In this new system, a mCherry construct was introduced into a *Neurospora* strain containing a 6X-TetO array. The TetR repressor bound to the PRC2 complex (SUZ12) was then introduced into the strain with the 6X-TetO array. In the default state of this Tet-Off system, the tetR repressor remains bound to the tetO repeats, effectively inhibiting the expression of the mCherry construct. Upon the addition of tetracycline, the tetR repressor dissociates from the tetO repeats, thereby allowing for the activation of the mCherry reporter.

Targeting PRC2 is sufficient for gene silencing at an artificial domain

The 6x-tetO array was introduced randomly in the *Neurospora* genome, and the first step was to determine if mCherry was being expressed in this strain. We made a conidial suspension of the strain and checked for the expression of mCherry using fluorescence microscopy. We observed robust expression of mCherry localized within the nuclei in the host strain (Figure 1A). Next, we wanted to know if mCherry expression would be affected if we targeted PRC2 to that gene locus. The mCherry gene was inserted next to the 6x-tetO array. We hypothesized that the binding of the tet-R-GFP-SUZ12 to the tetO-array would induce an ectopic H3K27me3 domain that would spread and silence the nearby mCherry gene. To confirm if this was true, we targeted the tet-R-GFP-SUZ12 protein to the tetO-array and checked to see mCherry expression was impacted in that strain. Again, we made conidial suspensions of the strain and checked for mCherry expression using fluorescence microscopy. We noticed a significant reduction in the

levels of mCherry fluorescence within the nuclei after PRC2 was targeted to that gene domain (Figure 1B). These results suggest that the presence of PRC2 at the domain is sufficient to repress the expression of the mCherry reporter gene.

An artificial H3K27me3 domain is established and spreads to nearby genes

Next, we wanted to confirm that H3K27me3 was deposited at the tetO-array and that it spread into the mCherry gene domain. We performed Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) on our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12). We investigated the patterns of H3K27me3 modifications in the wild-type (WT) strain and our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12). No H3K27me3 enrichment was detected at that domain in WT, but we observed a marked enrichment of H3K27me3 at the artificial tetO domains in our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12) (Figure 1C). This H3K27me3 modification spread beyond the initial nucleation site and into the mCherry gene. These results confirmed that the tethered PRC2 subunit was sufficient to establish an artificial H3K27me3. The H3K27me3 domain then spreads to nearby genes, leading to gene repression.

The artificial H3K27me3 domain is weakly maintained over time

Our final line of inquiry was to determine how well the artificial H3K27me3 domain is maintained. To test this, we added tetracycline to our growth media. The addition of tetracycline should trigger the dissociation of the tethered PRC2 complex from the tetO-array. Our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12) was grown in Vogel's minimal medium for 48 hours with tetracycline at a final concentration of 5 µg/ml (Figure 2A). We performed ChIP-seq to measure H3K27me3 enrichment at the targeted domain (Figure 2B). We found that there was still a little enrichment of H3K27me3. After 48 hours, we expected a 65,536 - 16 million fold dilution

of the H3K27me3 modification. The loss of H3K27me3 at this domain indicates that the methylation marks are being slightly maintained once PRC2 is no longer present. More work is needed to prove the mechanisms of maintenance in this system. We have demonstrated that targeting PRC2 is sufficient for repressing gene expression, as evidenced by the reduction of mCherry fluorescence in our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12). We also showed that once PRC2 is recruited, it deposits an artificial domain of H3K27me3, this domain spreads to nearby genes. These marks are slightly maintained for a little over 48 hours.

Discussion

Facultative heterochromatin can transition between active and inactive states, making it a crucial regulator of gene expression (Margueron & Reinberg, 2011b). The establishment and maintenance of facultative heterochromatin in mammals is primarily governed by two large multi-protein complexes known as Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2). PRC2 catalyzes the methylation of histone H3 at lysine 27 (H3K27me) across a variety of organisms, including plants, animals, and fungi (Chittock et al., 2017; Hennig & Derkacheva, 2009b; Z. A. Lewis, 2017; Ridenour et al., 2020). Multiple studies have focused on understanding how PRC2 is recruited and maintained in these organisms. We generated a reporter strain that allowed us to study the kinetics of *de novo* H3K27me3 establishment, spreading, and maintenance at an artificial domain. We reported that *de novo* establishment of H3K27me3 at a target site is sufficient for gene repression, as shown by the reduction of mCherry in our reporter strain (::6xTetO – mCherry ::tetR-GFP-SUZ12). These results support previous results reported in mammalian cells and yeast (Højfeldt et al., 2018; Ragunathan et al., 2015). We also showed that H3K27me3 spreads beyond the nucleation site and into neighboring genes. Our results further support the notion that PRC2 not only establishes H3K27me3 but also

facilitates propagation across neighboring histones via its “read-write” mechanism (Brickner, 2023). The established H3K27me3 domains were poorly maintained in the absence of PRC2. This suggests a model of maintenance that is independent of PRC2 recruitment. The observed spreading of H3K27me3 into neighboring genes raises important questions about the boundaries and regulatory factors that govern heterochromatin regulation in *Neurospora*. These results suggest that the influence of PRC2 extends beyond the immediate vicinity of its binding site and may impact gene expression across broader genomic regions. The mechanisms that regulate the spreading of H3K27me3 are poorly understood.

The unregulated spreading of heterochromatin inherently poses a risk to adjacent euchromatin due to its ability to influence neighboring chromatin structures. As heterochromatin expands beyond its boundaries, it can encroach upon euchromatin regions, leading to transcriptional repression and the silencing of nearby genes that need to be active. An example of this phenomenon is observed in *Drosophila melanogaster*, where the rearrangement of chromosomes places the white gene near pericentric heterochromatin, leading to its stochastic silencing in some cells (Elgin & Reuter, 2013). This phenomenon leads to great consequences during development because the precise expression of genes in euchromatin is critical for proper cellular function and identity. The spreading of heterochromatin to the wrong domains also influences genomic instability, potentially contributing to diseases such as cancer (Allshire & Madhani, 2018; Geutjes et al., 2012; Sharma et al., 2010). In our reporter strain, we noticed that the *de novo* H3K27me3 domain spreads beyond the site of nucleation, but we do not know why it does and if there are any boundaries regulating the spread of H3K27me3 in *Neurospora*. This new reporter strain provides us with a new tool for unraveling the mechanisms that restrict heterochromatin spread in the *Neurospora* genome.

In *Neurospora*, heterochromatin spreading is dominated by the H3K9me/HP1 system (Freitag et al., 2004; Honda & Selker, 2008; Jamieson et al., 2016). Trimethylation of histone H3 at lysine 9 (H3K9me3) is initiated by the recruitment of the histone methyltransferase DIM-5 complex (DCDC) (Z. A. Lewis et al., 2010b). H3K9me3 spreads to cover large gene domains once nucleated. Several factors have been identified that regulate the spreading of H3K9me3 in *Neurospora*. Histone demethylase LSD1 and DNA Methylation Modulator 1 (DMM-1) are crucial in maintaining H3K9me3 boundaries and preventing the spread of heterochromatin into euchromatin domains (Honda et al., 2010; Storek et al., 2020). Genome-wide analyses also demonstrated the general roles of H3K56 deacetylation and H2A.Z deposition in the aberrant spreading of heterochromatin (C. Zhang et al., 2022). Very little is known about the mechanisms that regulate the spreading of H3K27me3 in *Neurospora*.

A future direction for this study would be to use our reporter system to understand how H3K27me3 spreading is regulated in *Neurospora*. We will accomplish this by integrating additional 6x-tet-O arrays into various genomic locations to test if we can identify the boundaries that regulate the spreading of H3K27me3 domains. We hypothesize that the exist genomic boundaries that modulate the spreading distance of H3K27me3 domains. To test this hypothesis, we will create ten different strains featuring a 6x-tet-O array integrated at a random site within the genome. We will streak multiple 6x-tetO-mCherry primary transformants that have the 6x-tetO inserted randomly into the genome to obtain a homokaryon. We will then introduce the tet-R-GFP-SUZ12 construct into those strains and use fluorescence microscopy to determine if integration into different sites in the genome affects the spreading of H3K27me3. This workflow (Supplementary Figure 5) will enable us to identify any genomic features that define heterochromatin boundaries in *Neurospora*. We are also interested in identifying associated

proteins that contribute to the spreading of H3K27me3. This will be accomplished by creating mutations or deletions in specific genes coding for proteins known to interact with PRC2 or those that are implicated in chromatin remodeling. The results presented here and the proposed experiment should help broaden our understanding of the mechanisms controlling H3K27me3 establishment, spreading, and maintenance in *Neurospora crassa*.

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Figures

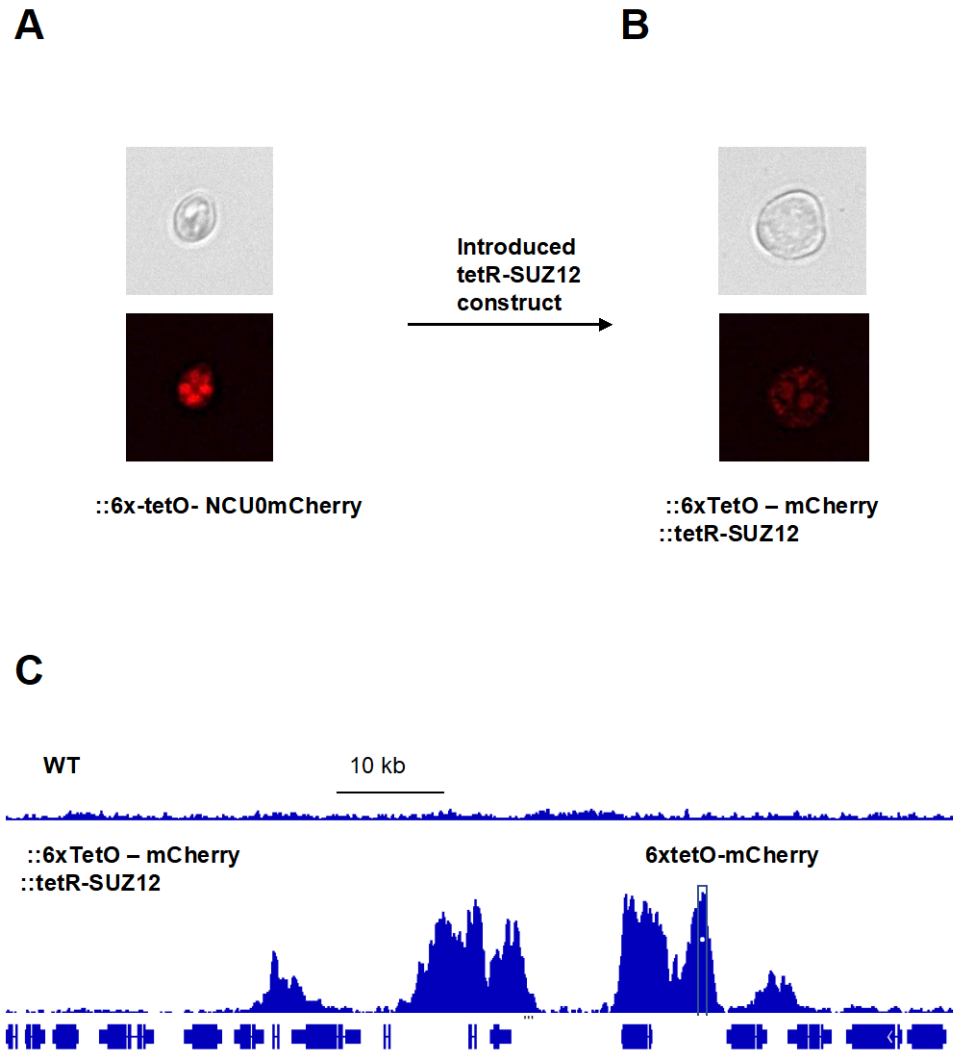


Figure 1: H3K27me3 is sufficient for gene repression. (A-B) Representative images of mCherry expression in the 6x-tetO-NCU0mCherry strain before and after the addition of tetR-GFP-SUZ12. (C) H3K27me3 enrichment from ChIP-seq in wildtype (WT) and our reporter strain (6xTetO – mCherry ::tetR-GFP-SUZ12). Zoomed in view of the target region where the 6x-tetO repeats were inserted into the *Neurospora* genome.

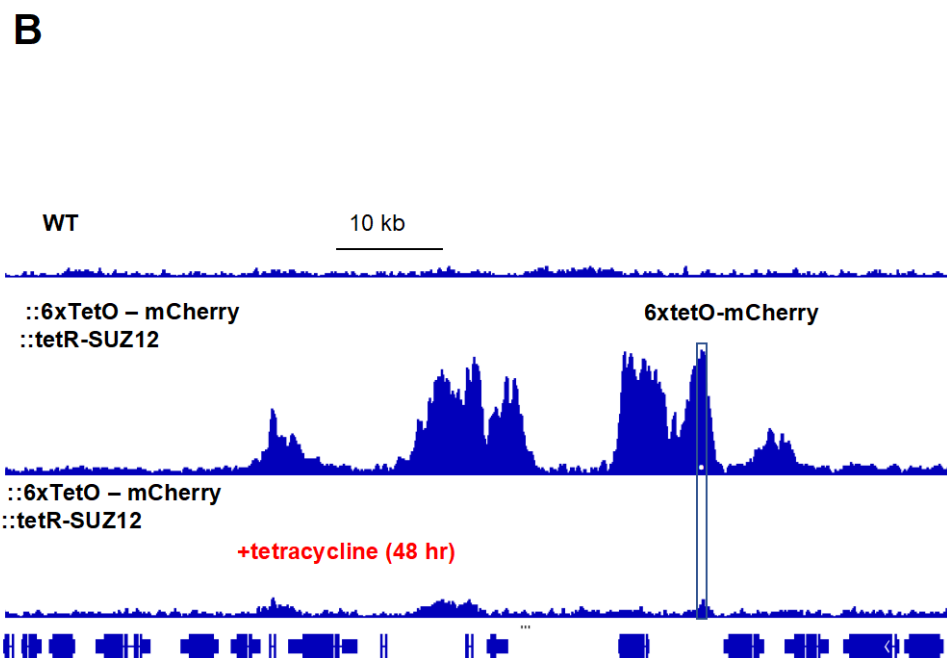
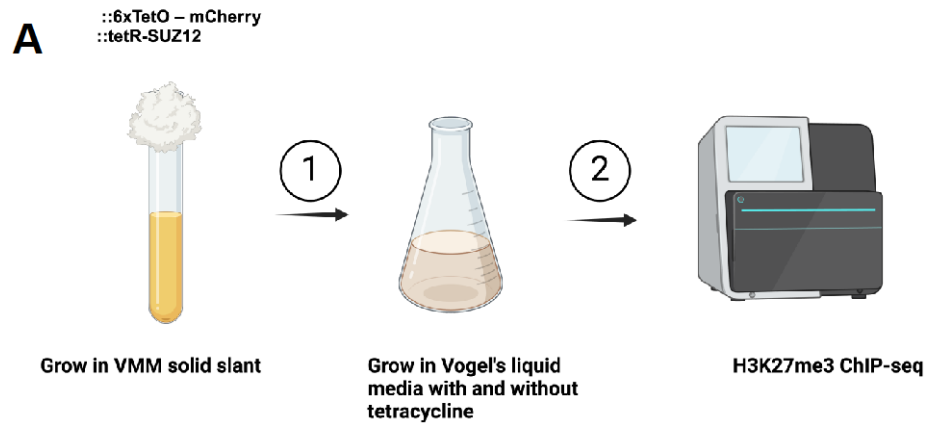
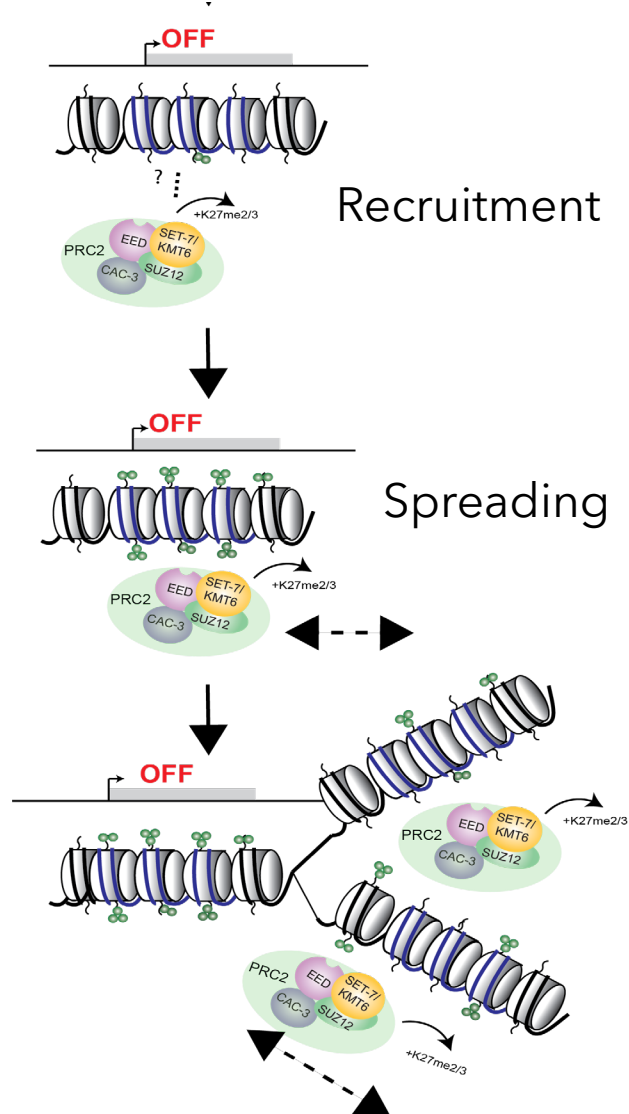


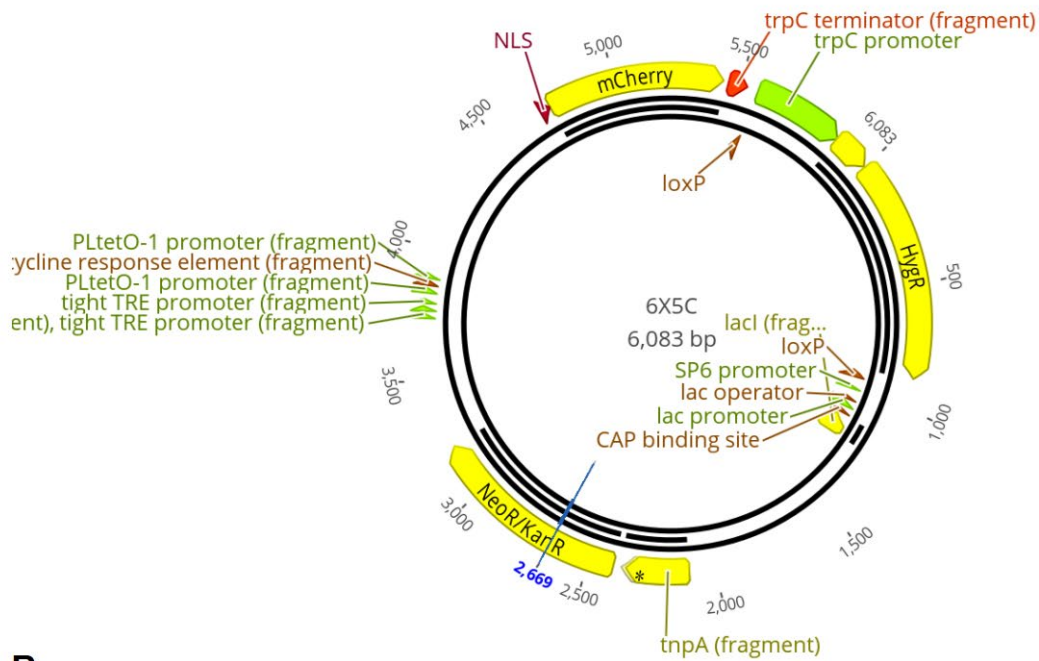
Figure 2: H3K27me3 is lost following tetracycline addition. (A) Cartoon of our experimental design. Our reporter strain was grown in Vogel's media plus tetracycline and ChIP-seq was used to measure H3K27me3 enrichment at the target-domain. (B) We performed ChIP-seq to measure H3K27me3 enrichment at the tetO array following the dissociation of tetR-GFP-SUZ12 from the target domain. We measured H3K27me3 enrichment in wildtype (*WT*) grown with no tetracycline and our reporter strain (*6xTetO – mCherry ::tetR-GFP-SUZ12*) grown with and without tetracycline.

Supplementary Figures

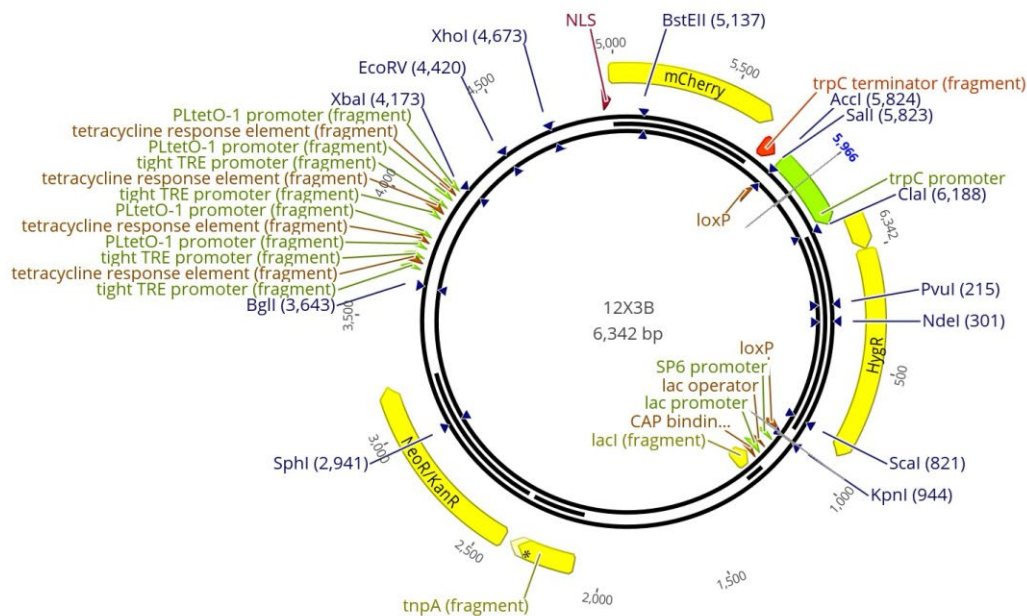


Supplementary Figure 1: Lab model of facultative heterochromatin assembly and maintenance. We proposed this model to explain how we think H3K27 methylated domains are assembled and maintained in *Neurospora crassa*. The assembly and maintenance of H3K27 methylation are two separate processes. First, Polycomb Repressive Complex 2 (PRC2) is recruited and binds naïve chromatin. The chromatin domain is methylated with H3K27. The H3K27 methylation spreads beyond nucleation site via PRC2 “read-write” mechanism. Finally, the H3K27 methylation is maintained and passed down over multiple rounds of cell divisions.

A



B

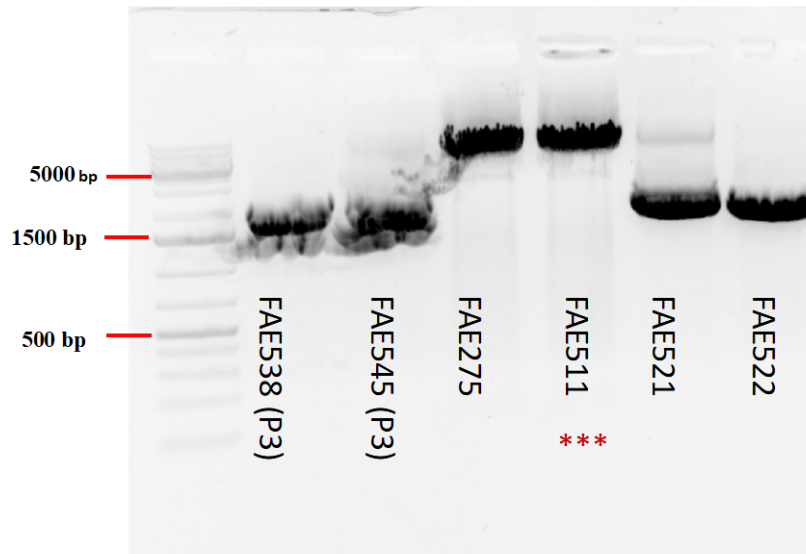


Supplementary Figure 2: 6x-tetO repeats and 12x-tetO repeats plasmid maps. Each plasmid was made up of 6-12 tetO arrays. An mCherry gene was inserted near the array to test if the recruitment of PRC2 to the tetO array could lead to the silencing of the mCherry gene. Each plasmid also contained a Nuclear Localization Signal (NLS) to target the construct into the nucleus. The HYG cassette was used as a drug-selection to select for primary transformants. **(A)** 6x-tetO array plasmid. **(B)** 12x-tetO array plasmid.

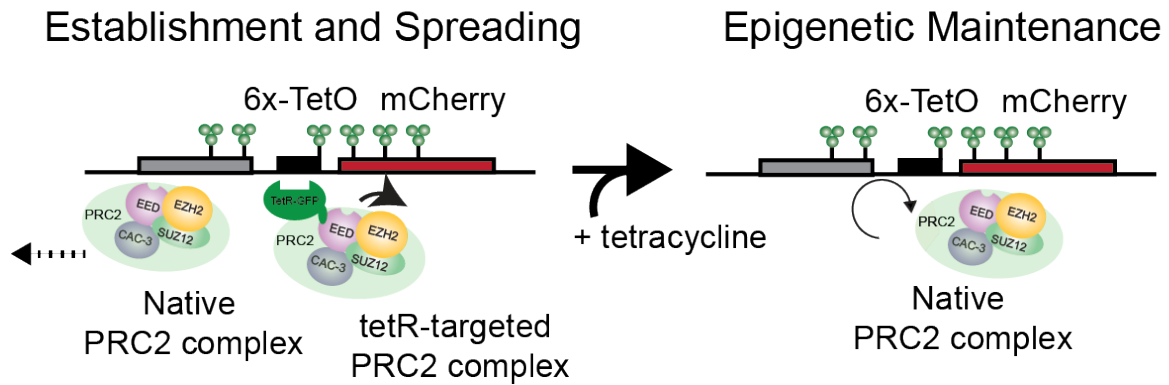
A

Strain	Genotype
FAE538 (P3)	6X crossed to S2
FAE545 (P3)	12X crossed to S2
FAE275	Original tet-R-SUZ12
FAE511	Tet-R-SUZ12;6X tet O
FAE521	Tet-R-SUZ12;12X tet O
FAE522	Tet-R-SUZ12;12X tet O

B

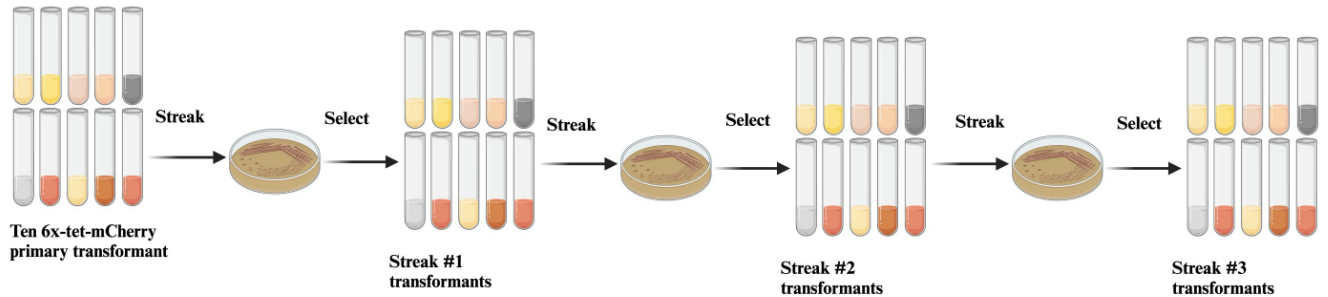


Supplementary Figure 3: *csr-1* genotyping of Tet-R-GFP-SUZ12; tet O strains. We extracted genomic DNA from each of the strains listed in the table and performed PCR to confirm that the tetR-GFP-SUZ12 construct was inserted into the *csr-1* locus of our host strains (FAE538 and FAE545). **(A)** Table showing all the strains that were genotyped. **(B)** PCR results of electrophoresis gel. Strain FAE511 had the correct size insertion at the *csr-1* locus corresponding to the insertion of the tetR-GFP-SUZ12 construct.

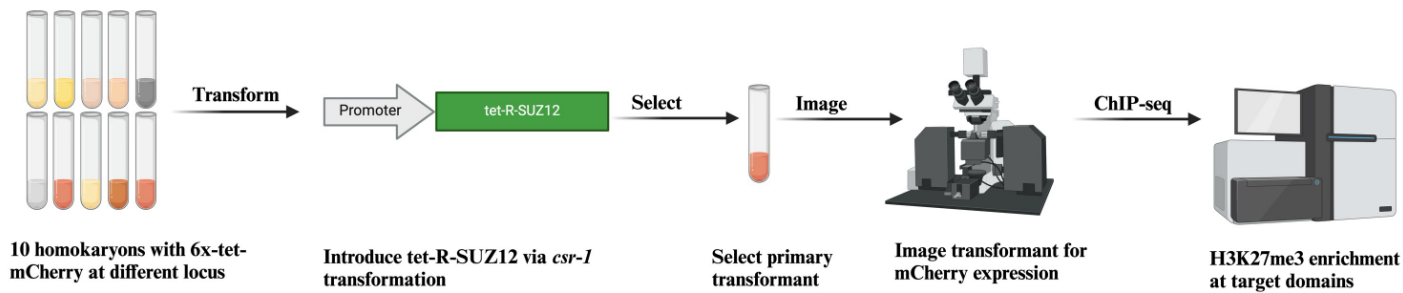


Supplementary Figure 4: Cartoon of *tetO/tetR* experimental design. Schematic cartoon of TetR-GFP-PRC2 at the *csr-1::6xTetO-mCherry* locus. Tetracycline (tet) promotes the release of the TetR-targeted-PRC2 complex from *tetO* sites. We can measure establishment and maintenance independently by looking at the expression of the *mCherry* gene.

Part 1: Streak on HYG plates to obtain homokaryons



Part 2: Introduce tet-R-SUZ12 construct into homokaryons



Supplementary Figure 5: Workflow to examine H3K27me3 spreading mCherry in the reporter strain. We will streak ten different primary transformants from our transformation, where we introduced the 6x-tetO-mCherry construct randomly into the genome of our wildtype strain (S2). These strains will be streaked successively to obtain a homokaryon. For the second part of the experiment, we will introduce the tet-R-GFP-SUZ12 construct into the *csr-1* locus of the ten homokaryon strains with the 6x-tetO-mCherry. We will use fluorescence microscopy and ChIP-seq to identify boundaries involved in spreading H3K27me3 in *Neurospora*.

Tables

S1: Strains used in this study

Genotype	Strain #	Reference
<i>Wildtype</i>	S2	
<i>6xTetO – mCherry</i>	FAE538	This study
<i>6xTetO – mCherry ::tetR-GFP-SUZ12</i>	FAE511	This study

S2: Primers used in this study

Name	Sequence (5'-----3')
Csr-1 genotype fwd	GATGCCAAGAAATCCCGGGA
Csr-1 genotype rev	TCGCCAACACATTGAATGCT