THE ROLE OF CHROMODOMAIN PROTEIN 1 (CDP-1) AT *NEUROSPORA CRASSA* HETEROCHROMATIN

by

CARISSA KIM

A THESIS

Presented to the Department of Biology
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2016

An Abstract of the Thesis of

Carissa Kim for the degree of Bachelor of Science
in the Department of Biology to be taken June 2016

Title: The Role of Chromodomain Protein 1 (CDP-1) at *Neurospora crassa* Heterochromatin

Approved: 

Eric U. Selker

Proper heterochromatin[1] formation is a significant factor in chromosome organization and gene regulation. Therefore, it is vital to study the role of all proteins that contribute to proper heterochromatin formation in order to form a comprehensive understanding of the importance of heterochromatin. In *Neurospora crassa*[2], heterochromatin formation depends on a number of well-studied proteins. However, we still have an incomplete understanding of this process.

In order to improve our understanding of heterochromatin formation in *N. crassa,* the Selker lab has identified many novel proteins involved in this process. One novel protein is chromodomain protein 1 (CDP-1) that specifically localizes to heterochromatin. We hypothesized that CDP-1 is involved in proper heterochromatin formation. To begin understanding the role of CDP-1 at heterochromatin, we asked:

[1] Heterochromatin—generally, it is a tightly packed form of chromatin (organizes DNA) that can (though not always) prevent certain proteins from accessing certain regions of DNA
[2] *Neurospora crassa* (*N. crassa*)—fungal species used in the Selker lab, as well as other labs, as a genetic model

1) Is CDP-1 required for proper heterochromatin formation at specific regions, such as the centromeres, telomeres, and interspersed heterochromatic regions?
2) Which domains[3] of CDP-1 are required for proper protein function?
3) Do changes in gene expression occur when the *cdp-1* gene is deleted?
4) Do proteins or protein complexes associate with CDP-1? If so, do they play a role in CDP-1 function?

To test if CDP-1 is required for proper silencing at heterochromatic regions, my mentor knocked out[4] the *cdp*-1 gene and inserted antibiotic resistance genes at various heterochromatic regions (centromere on linkage group[5] I and VI, a telomere on linkage group VII, and in interspersed heterochromatin). I screened strains for the antibiotic resistance genes and the knockouts of certain genes of interest (e.g. *cdp-1*, *hpo*[6]) using Southern blots[7]. I tested the growth of the strains on antibiotic media to determine if heterochromatin was still forming properly at these regions with *cdp-1* deleted.

Once I determined where CDP-1 is required for proper heterochromatin formation, I tested which domains of CDP-1 are required for CDP-1 function. I transformed[8] a *cdp-1* knockout strain with plasmids[9] that contained the *cdp-1* gene with

[3] Domains—proteins contain amino acid sequences which form a three-dimensional structure that carries out specific functions for the protein (catalytic, binding). CDP-1 has 2 types of domains: a chromodomain that allows CDP-1 to recognize and bind to chromatin and AT hooks that help CDP-1 bind to AT-rich DNA regions.
[4] Knocked out/knockout—deletion (or replacement) of gene from genome
[5] Linkage group—a chromosome in *N. crassa*
[6] *hpo*—a gene encoding the protein, HP1, in *N. crassa* that is essential for heterochromatin formation. It is a highly conserved protein in higher organisms.
[7] Southern blot—assay to detect the placement of restriction sites around a specific DNA sequence. See methods.
[8] Transformed/Transformation—genetic technique to insert a specific gene into the genome of an organism. See methods.

specific point mutations (chromodomain (CD), AT hook 1, AT hook 2, and AT hook 1/2 double mutant)[10]. These strains also had antibiotic resistance genes inserted into the genome (CenVIR::bar or CenIL::nat-1). I will test the CDP-1 mutant strains for growth on antibiotic media and antibiotic resistance gene expression using qRT-PCR.

RNA sequencing[11] (RNA-seq) showed that there were some genes whose expression levels changed when *cdp-1* was deleted. Based on the RNA-seq results, I focused on the expression of six different genes to confirm the data from the RNA-seq. We confirmed that one gene, NCU16718[12], had significantly higher expression levels when *cdp-1* was deleted compared with wild type. This gene is located in the sub-telomeric region of linkage group III, indicating that CDP-1 is required for silencing of this gene at telomere IIIR.

Finally, to determine if CDP-1 interacts with other proteins or protein complexes *in vivo,* I examined four different proteins that immunoprecipitated with CDP-1 in a large scale pull-down assay performed by a former member of the Selker lab. The four proteins (referred to as interacting partners from here on) that I am interested in are CRF8-1, SPT-16 and POB-3 (FACT complex), and HIR-1 (HIRA complex). Each interacting partner has a role at chromatin and potentially associates with CDP-1 in *N. crassa*. I tagged[13] all four interacting partners with 3xHA::hph[14] and crossed[15] them to

---

[9] Plasmid—circular bacterial DNA that can be manipulated for genetic experiments, such as transformations.
[10] Point mutations—mutations in the DNA sequence that alters one nucleotide into another nucleotide changing the amino acid sequence of the protein encoded by the DNA.
[11] RNA sequencing—technique to measure RNA levels
[12] NCU16718—a hypothetical gene in *N. crassa* genome whose function has yet to be defined.
[13] Epitope tag—a nucleotide sequence genetically inserted at the end of a gene that is translated with the gene and can be detected with antibodies
[14] 3xHA::hph—3 sequence repeats of HA tag that can be detected with HA antibodies. The *hph* antibiotic resistance gene is used for the selection of the tag [11]

another strain with 3xflag::hph[16] tagged CDP-1. I screened for strains with both CDP-1 and interacting partners tagged as well as strains with only interacting partners tagged. I then performed a Co-immunoprecipitation (Co-IP)[17] to determine if each interacting partner is pulled down with CDP-1 when CDP-1 is immunoprecipitated.

My results demonstrate that CDP-1 is required for silencing at centromere I and VI, as well as telomere IIIR. However, CDP-1 is not required for silencing at telomere VIIL or at interspersed heterochromatic regions. In addition, my results demonstrate that CDP-1 may interact with other proteins in order to carry out its function at heterochromatin. Further testing of the association between CDP-1 and the interacting partners, as well as, further efforts to produce stable mutant CDP-1 proteins will expand our understanding of CDP-1 function at heterochromatin. However, the preliminary findings that CDP-1 is required for proper heterochromatin formation at only certain heterochromatic regions in *N. crassa* contribute to our understanding of heterochromatin.

---

[15] Crossed—2 *N. crassa* strains were mated, see methods
[16] 3xflag::hph—similar to 3xHA::hph, repeated sequence for flag tag that can be detected with flag antibodies[11]
[17] Co-immunoprecipitation—target protein co-precipitates a protein partner, see methods.

Acknowledgements

I would like to thank Dr. Tish Wiles for her outstanding mentorship throughout my time in the Selker lab, for the support in my academic endeavors, and for challenging me to think and learn like a successful scientist. I would also like to thank Dr. Eric Selker for allowing me to be a part of his lab and helping me in the thesis process. Thank you to both Dr. Wiles and Dr. Selker for supporting my work and providing invaluable advice. I would also like to thank all the members of the Selker lab for the constructive input on my thesis and general help in the lab.

I would also like to thank my family, my parents and brother, for supporting my academic career and allow me to pursue my interests. I would also like to thank my family for supporting me these past three years emotionally and financially.

Table of Contents

Introduction

Background: Heterochromatin

What is heterochromatin?

Most eukaryotic[18] metazoan[19] organisms (e.g. animals) are made up of billions of cells. The nucleus of each cell carries DNA encoding the necessary information for cell function. Each human cell holds about six feet of DNA that needs to fit into a microscopic nucleus. This is achieved by organization and compaction of the DNA into chromatin. One-hundred forty-six base pairs of DNA is wrapped around a complex of eight proteins, each called a histone[20], to form nucleosomes[21] (Figure 1). There are hundreds of thousands of nucleosomes within a nucleus and individual histones can be modified through specific biochemical processes to form two general types of chromatin: euchromatin and heterochromatin. Euchromatin is generally thought to be loosely packed chromatin that is transcriptionally active. In contrast, heterochromatin is generally thought to be densely packed, which may prevent the access of transcription factors[22] to DNA, making heterochromatin relatively transcriptionally inactive or silent [1]. Heterochromatin is found in specific regions of chromosomes (generally where there are few transcriptionally active genes), specifically in regions such as the centromere[23], telomere[24], and select interspersed regions across a chromosome. These

[18] Eukaryotic—organism whose cells contain a nucleus, membrane-bound organelles, and cytoskeleton. Both uni- and multicellular.
[19] Metazoan—multicellular organism
[20] Histone—a protein that associates with DNA and has tails of amino acids
[21] Nucleosome—one set of eight histones with DNA wrapped around it
[22] Transcription factors—proteins that assist in DNA transcription into RNA
23 Centromere—region of chromosome. It separates the long arm from the short arm on the chromosome and is attached to spindles during mitosis and meiosis.

heterochromatic regions have distinguishable marks that we can examine experimentally.

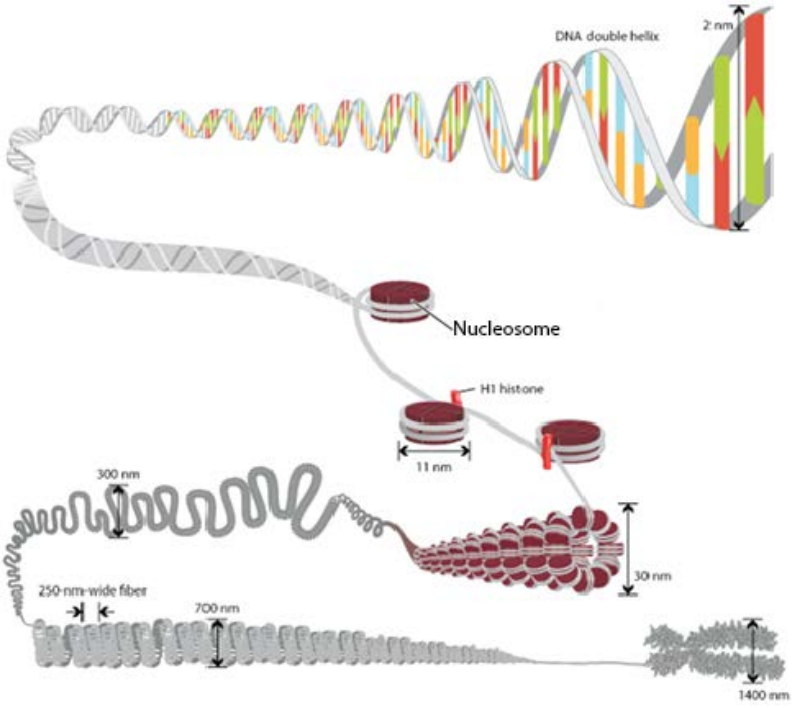

Figure 1: How DNA is organized.

DNA is wrapped around an octamer of histones to form nucleosomes. The nucleosomes form chromatin and are eventually organized to form chromosomes. Modified from Nature Education 2014 [4]

Heterochromatin has distinct biochemical markers

Heterochromatin is distinguished through certain biochemical modifications. One modification is histone tail methylation where a methyl group ($-CH_3$) is added to specific amino acids on the histone tails. The histone tails are long chains of amino acids; the methylation of amino acid lysine number 9 on histone H3 (H3K9me), typically marks heterochromatin. This amino acid residue can have 1-3 (mono-, di-, and

[24] Telomere—region at the ends of chromosome. Consists of a series of repeated DNA sequences and helps to stabilize the chromosome

tri-) methyl groups, though the di- and tri- forms are generally associated with heterochromatin. In addition, methylation of numerous other amino acids are found on histone tails at heterochromatin (Figure 2) [2]. Histone methylation can guide proteins to heterochromatic regions and lead to processes required for proper heterochromatin formation such as DNA methylation, another type of modification that is abundant in some types of heterochromatin. DNA methylation is the process of adding methyl groups to certain cytosines[25] in DNA. Constitutive[26] heterochromatin in *N. crassa* has both DNA methylation and H3K9me3.

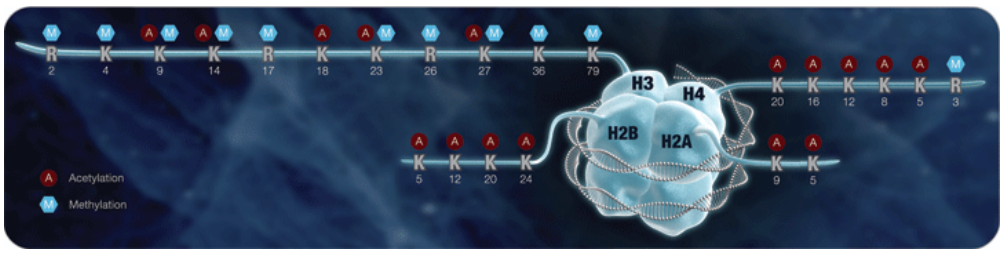

Figure 2: Biochemical markers on histone tails.

Nucleosome—there are four distinct histones: H2A, H2B, H3, and H4; in a nucleosome (two of each). Each histone has a tail that can be chemically modified—acetylated and methylated. SABiosciences 2008 [2]

Why is heterochromatin important?

Heterochromatin plays a role in the organization of chromosomes. Heterochromatin is prominently located in centromeres and telomeres and is important for stabilizing chromosomes. At the centromere, heterochromatin is required during cellular processes, such as mitosis[27]. Heterochromatin is important for recruiting

[25] Cytosine—one of four nuclei acids that comprises DNA
[26] Constitutive heterochromatin—chromatin that remains condensed throughout the cell cycle [1]
[27] Mitosis—process in cells that replicates DNA and splits one cell into two new genetically identical cells

proteins like cohesin[28], which is vital to hold sister chromatids together during certain stages of mitosis [1]. Heterochromatin also helps recruit kinetochore[29] proteins to centromeres (Figure 3). Without heterochromatin, chromosomes in mitosis would not segregate properly between daughter cells, thus leaving cells with an improper number of chromosomes—resulting in serious genetic complications and death. Overall, heterochromatin has an important function in chromosome segregation by recruiting essential proteins.

[28] Cohesin—adhesion molecule
[29] Kinectochore—protein that helps connect the centromere to microtubules during mitosis and meiosis

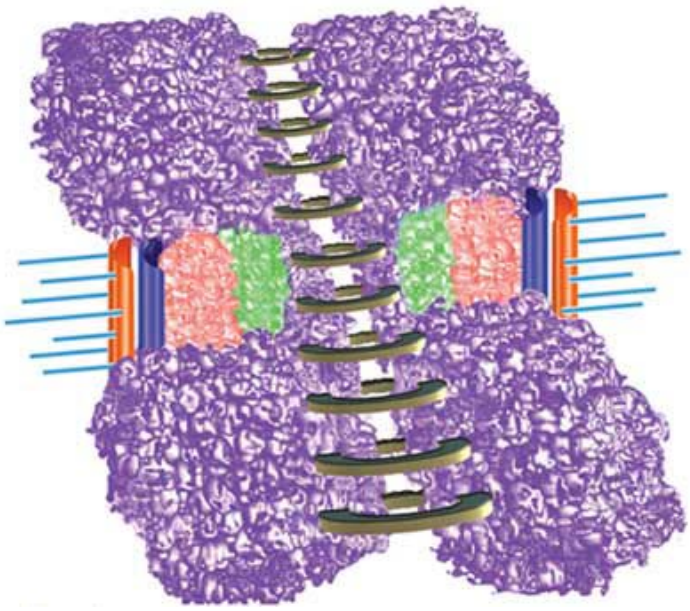

Figure 3: Schematic of chromosome interactions with cellular process proteins.

Chromosome—the centromere (red and green) contains heterochromatin that recruits kinetochores (dark blue and orange) and attaches microtubules (light blue). Heterochromatin throughout the chromosome also recruits cohesins (gray) that holds the two sister chromatids together (purple). Modified from Sullivan & Karpen 2004 [5]

Background: Neurospora crassa

N. crassa as an ideal model organism

N. crassa is an orange filamentous fungus that is eukaryotic. It is primarily haploid throughout its life cycle, meaning that it only has one copy of every chromosome (humans have 2 copies). Since *N. crassa* is haploid, genetic experiments are more direct as there is no second copy of genes confounding results. More specifically, having one copy of a gene allows scientists to easily observe the effects of deleting that gene and not have an extra, working copy, in the genome. *N. crassa* is also very easy to manipulate and propagate in a laboratory setting. Like other model

organisms, *N. crassa* is amenable to perform genetic experiments on, such as mutagenesis[30] and complementation tests[31] [3].

N. crassa is excellent for studying heterochromatin

There are many other organisms that researchers use to study epigenetics[32] and heterochromatin. However, *N. crassa* is a more suitable organism in some epigenetic studies. First of all, *N. crassa* contains certain heterochromatic markers, such as DNA methylation, whereas certain model eukaryotes, such as, *Caenorhabditis elegans* (*C. elegans*) and yeasts (*S. cerervisiae* and *S. pombe*), lack DNA methylation [3]. Higher eukaryotes, such as humans and mice, have DNA methylation; however, it is difficult to study because it is essential to organismal viability [3]. As a result, *N. crassa* is an ideal organism to study epigenetics and heterochromatin because it is more similar to humans than other widely studied model organisms.

Background: Chromodomain Protein 1 (CDP-1)

A model for heterochromatin formation in N. crassa.

In *N. crassa* there are many proteins that are required for proper heterochromatin formation and the Selker lab has created a model for heterochromatin formation in *N. crassa* (Figure 4) [3]. First, the DCDC complex[33] is recruited to AT-rich DNA[34]. DIM-7 of the DCDC complex directs DIM-5[35], a histone methyltransferase, to

[30] Mutagenesis—process that changes/mutates the genetic information (DNA)
[31] Complementation test—test to determine if mutations in two strains are in different genes and can rescue the functional/working phenotype
[32] Epigenetics—the study of heritable changes in gene expression and function
[33] DCDC complex—a protein complex comprise of proteins, DIM-5, DIM-7, DIM-9, DDB-1, and CUL4
[34] AT-rich DNA—in *N. crassa*, heterochromatic regions are adenine (A) and thymine (T) rich due to genetic modifications

heterochromatin to methylate H3K9 [6]. Once H3K9 is methylated, heterochromatin protein 1 (HP1)[36] is able to be recruited to heterochromatin. The recruitment of HP1 subsequently recruits a number of proteins and protein complexes to heterochromatin, such as DIM-2[37], the HCHC complex[38], and the DMM complex[39]. DIM-2, a DNA methyltransferase, methylates cytosines in heterochromatin, the HCHC complex is responsible for deacetylating histones, and the DMM complex prevents spreading of heterochromatin into transcriptionally active regions. Together, these proteins and protein complexes work to establish specific types of heterochromatin in *N. crassa*.

[35] DIM-5—a histone methyltransferase in *N. crassa* that methylates lysine 9 on histone H3 (H3K9)
[36] HP1—a chromodomain protein that is highly conserved in organisms. In *N. crassa* it binds to H3K9me and facilitates binding of other proteins at heterochromatin
[37] DIM-2—a DNA methyltransferase in *N. crassa* that methylates cytosines (5mC)
[38] HCHC complex—a complex of proteins whose main function is to remove acetyl groups from histones
[39] DMM complex—a complex of proteins that prevents spreading of heterochromatin into nearby euchromatic regions

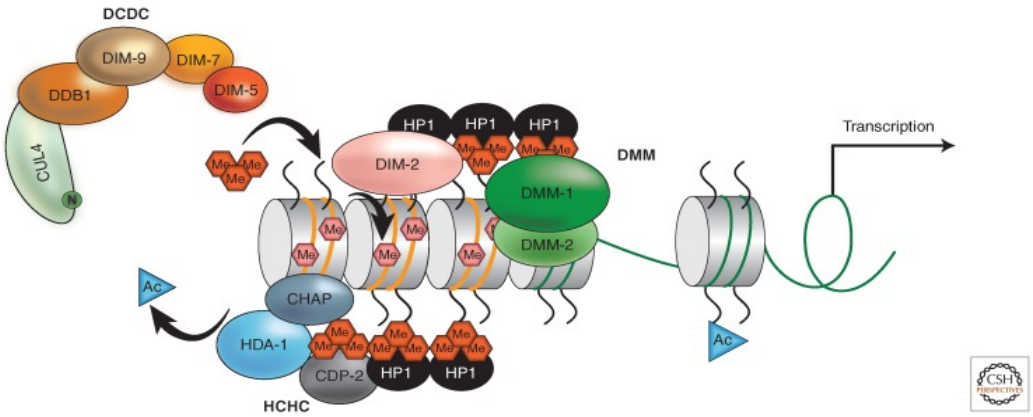

Figure 4: Model of heterochromatin formation in *N. crassa*.

The DCDC, HCHC, and DMM complex in addition to the proteins HP1 and DIM-2 function together to form heterochromatin in *N. crassa* in the appropriate regions. Aramayo & Selker 2013 [3]

CDP-1 is a chromodomain protein and may not function in the current heterochromatin assembly model

Similar to some of the proteins in this model (e.g. HP1, CDP-2), CDP-1 has a chromodomain (Figure 5). The chromodomain allows CDP-1 to recognize and bind to H3K9me. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) data shows that CDP-1 localizes to regions with H3K9me3 (Figure 6). CDP-1 also has two AT hook domains (Figure 5). AT hooks are small DNA-binding motifs that bind to AT-rich DNA in the minor groove[40] of DNA using a conserved nine amino acid sequence[41] [7]. CDP-1 AT hooks could be a potential mechanism for how CDP-1 localizes to AT-rich DNA in *N. crassa* heterochromatin.

[40] Minor groove—double helix DNA has a major (larger) and minor (smaller) groove. Proteins can bind to either major or minor groove

[41] Amino acid sequence—after DNA is replicated and RNA is translated, the product is an animo acid sequence that make up proteins

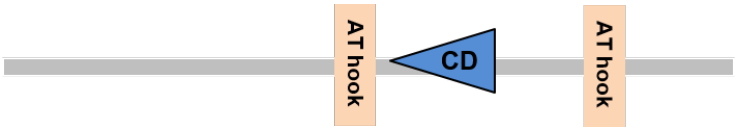

Figure 5: Protein layout of CDP-1 based on DNA sequence.

This shows a 2-dimensional layout of CDP-1 domains. CDP-1 has a chromodomain (CD) and two AT hook domains.

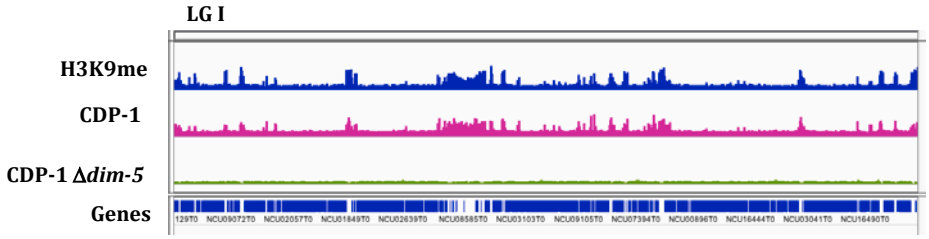

Figure 6: ChIP-seq data at LG I with gene layout.

Top track (blue): the peaks show where H3K9me3 is located across LG I. Middle track (pink): the peaks show where CDP-1 localizes to on LG I. The localization of CDP-1 overlaps with H3K9me3. Bottom track (green): shows CDP-1 localization when *dim-5* is deleted. Without DIM-5, H3K9 is not methylated and CDP-1 cannot localize to heterochromatin, via H3K9me3. (Ref. Tish—personal communication)

It is unclear how CDP-1 contributes to heterochromatin formation as depicted in the model (Figure 4). However, CDP-1 is required for gene silencing at Centromere VI (CenVI) in *N. crassa* (Figure 7). When CDP-1 was knocked out and antibiotic resistance genes were inserted into CenVI, CDP-1 knockout strains were able to grow on antibiotics, suggesting that the antibiotic resistance gene is expressed and heterochromatin is not forming properly. As a result, CDP-1 has some role in heterochromatin formation in *N. crassa* that my research will investigate.

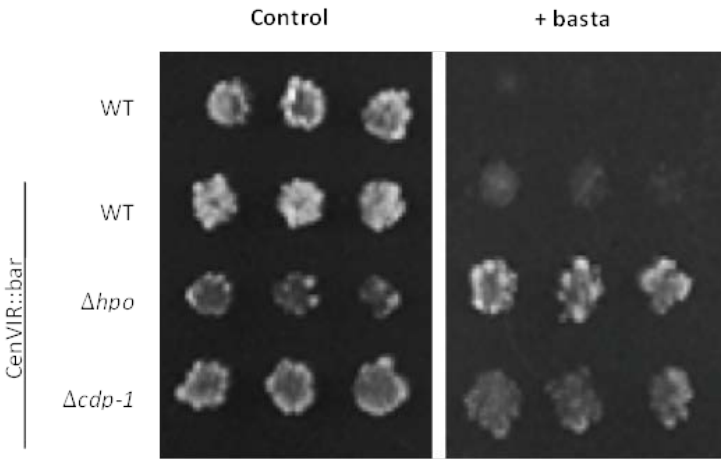

Figure 7: Spot test silencing assay for Centromere VI.

The left of the figure shows the plate that had no drug; the one on the right had the drug Basta added. The bottom three strains have the *bar* gene inserted. The strains that were able to grow on Basta were expressing the *bar* gene and subsequently had improper heterochromatin formation.

Hypothesis

CDP-1 is required for proper heterochromatin formation

Chromodomain Protein 1 (CDP-1) is required for some heterochromatic silencing in *N. crassa*. It is specifically required for silencing at the centromere on linkage group (LG) VI (CenVI). This was concluded from a spot test silencing assay where a *bar* antibiotic resistance gene was inserted at CenVI (Figure 7). If heterochromatin forms properly at the centromere, the inserted *bar* gene will be silenced. In the absence of the CDP-1 gene (*cdp-1*) the *bar* gene was expressed suggesting that heterochromatin formation at CenVI is disrupted. This initial finding prompted me to hypothesize that CDP-1 is required for silencing at heterochromatic regions—centromeres, telomeres, and interspersed heterochromatic regions.

CDP-1 chromodomain is required for CDP-1 function at heterochromatin

Based on prior research, CDP-1 localizes to heterochromatin, specifically to H3K9me3, and this may be attributable to CDP-1 chromodomain (CD). In other proteins, such as HP1, the CD is required for protein localization to heterochromatin; therefore, in CDP-1 I hypothesized that the CD is required for CDP-1 localization to and function at heterochromatin. I also hypothesize that the other domain of CDP-1, the AT hooks, are not required for CDP-1 localization to and function at heterochromatin.

CDP-1 affects gene expression

In order to have a comprehensive understanding of the importance of CDP-1 in *N. crassa*, I began to test if CDP-1 has any effect on gene expression. In a preliminary experiment, deleting *cdp-1* from the *N. crassa* genome caused changes in RNA

transcript levels of certain genes throughout the genome (data not shown). I wanted to study this effect further by obtaining a quantitative analysis of the changes in gene expression due to loss of CDP-1.

CDP-1 interacts with chromatin associated proteins (CRF8-1, POB-3, SPT-16, HIR-1) in N. crassa

A former Selker lab member performed a large scale pull-down assay with epitope tagged CDP-1 and found that there were many proteins that could interact with CDP-1 *in vivo*. In order to confirm these interactions, I conducted a Co-IP[42]. I hypothesized that CDP-1 will interact with all these proteins in *N. crassa*.

[42] See methods.

Results

CDP-1 is required for silencing at Centromere IL

CDP-1 was shown to be required for heterochromatic silencing at CenVI[43] (Figure 7). I wanted to know if CDP-1 is required for heterochromatic silencing at other centromeres as well. I began by testing the centromere on linkage group I (CenI). I inserted the *nat-1* antibiotic resistance gene into CenI and confirmed my insertion through Southern blot analysis (data not shown). To test if CDP-1 is required for silencing at CenI, I performed a spot test silencing assay (Figure 8). I used wild type (WT) (negative control), related WT with *nat-1* inserted, *hpo*[44] deletion with *nat-1* (positive control), and *cdp-1* deletion with *nat-1* strains. Both the WT and related WT (with *nat-1*) were not able to grow on antibiotic medium because heterochromatin is forming properly and silencing the resistance gene (Figure 8). Regardless of inserting the *nat-1* gene, heterochromatin is still forming properly in this strain. For a positive control, I used a *hpo* deletion strain; HP1/*hpo* is essential for heterochromatin to form properly. On the antibiotic medium, the *nat-1*/*hpo* deletion strain was able to grow because heterochromatin formation is disrupted and *nat-1* is expressed. Finally, I used a *cdp-1* deletion strain with the *nat-1* antibiotic resistance gene inserted. This strain grew on the antibiotic medium suggesting that heterochromatin was not forming properly after deleting *cdp-1*. From this experiment, I concluded that CDP-1 is required for proper heterochromatin formation and heterochromatic silencing at CenI.

[43] Ref. Tish (personal communication)
[44] *hpo*—gene coding for heterochromatin protein 1 (HP1)

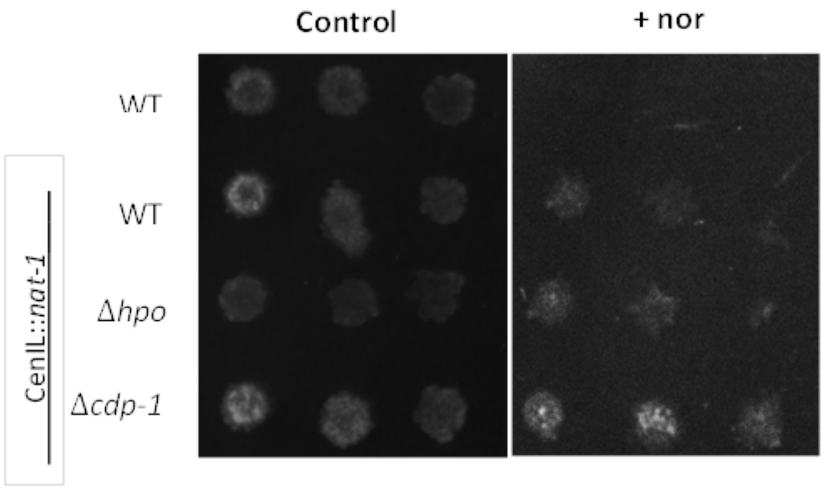

Figure 8: Spot test silencing assay for Centromere I.

The left of the figure shows the plate that had no drug; the one on the right had the drug nourseothricin (nor) added. The bottom three strains have the *nat-1* gene inserted. The strains that were able to grow on nor were expressing the *nat-1* gene and subsequently had improper heterochromatin formation.

CDP-1 is not required for silencing at telomere VIIL

I determined that CDP-1 is required for silencing at CenI in addition to CenVI. Next, I wanted to test if CDP-1 is also required for silencing at telomeres, where heterochromatin is also normally found. I used the same set-up as for the centromere spot test silencing assays, except using a strain with the *bar* gene at telomere VII (telVII) (Figure 9). The first two control strains that I used were not able to grow on the antibiotic medium presumably because heterochromatin was forming properly even when I inserted the antibiotic resistance gene *bar*. For a positive control, I used the *hda-1*[45] deletion strain instead of *hpo* because *hda-1* has been shown to be required for silencing, specifically at telomeres [13]. After deleting *hda-1*, the strain is able to grow on the antibiotic medium because heterochromatin formation at the telomere is

[45] *hda-1*—histone deacetylase, part of the HCHC complex; a protein that functions to remove acetyl groups from histone tails (markers of euchromatin)

disrupted. When I tested a *cdp-1* deletion strain with *bar* inserted in telVIIL, I found that the strain was unable to grow on antibiotic media. This finding suggests that CDP-1 is not required for silencing at telVII.

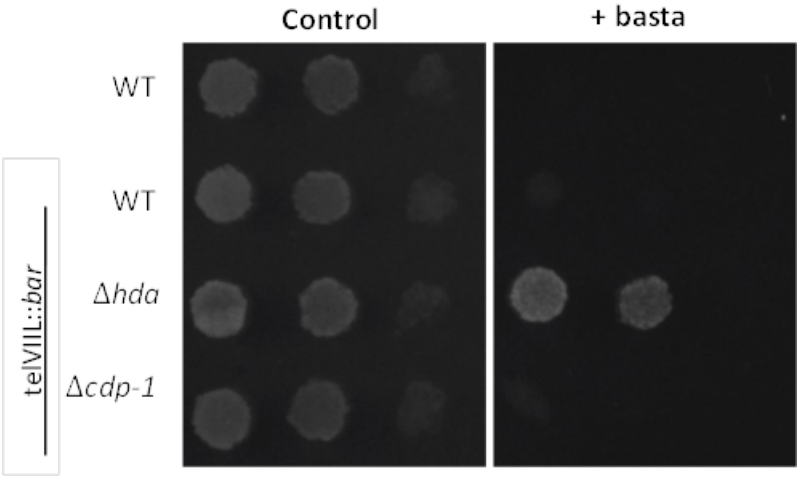

Figure 9: Spot test silencing assay for Telomere VII.

The left of the figure shows the plate that had no drug, the one on the right had Basta added. The bottom three strains have the *bar* gene inserted. The strains that were able to grow on Basta were expressing the *bar* gene and subsequently had improper heterochromatin formation.

CDP-1 is not required for silencing at interspersed heterochromatic regions

Finally, I looked at interspersed heterochromatic regions, i.e. heterochromatic regions that are located between the centromeres and telomeres. In interspersed heterochromatic regions, there may be genes that can be found and observed [14]. Specifically, I looked at heterochromatin formation at the *am* gene[46]. First, I used a strain that had the antibiotic resistance gene *hph* inserted in the *am* gene [15]. I again was testing if deleting *cdp-1* caused any changes in heterochromatin formation at the *am* gene. I used two controls (WT and a related WT with the antibiotic resistance gene);

[46] am gene—encodes protein for alanine synthesis. Through a specific genetic mechanism, in this strain, this gene is heterochromatically silenced in *N. crassa*

they both did not grow on the antibiotic medium. The positive control, an *hpo* deletion strain, was able to grow on antibiotic media presumably because heterochromatin formation was disrupted. When I deleted *cdp-1*, the strain was unable to grow on the antibiotic medium. This showed that CDP-1 is not required for heterochromatic silencing at interspersed heterochromatic regions.

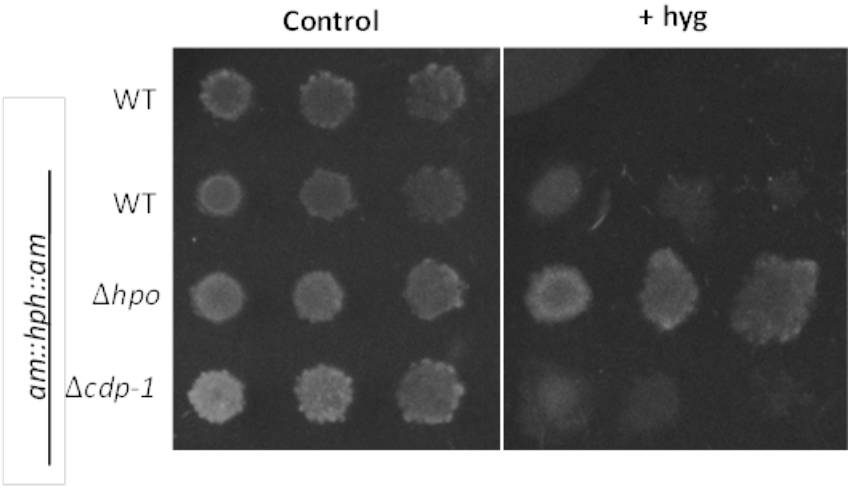

Figure 10: Spot test silencing assay for interspersed heterochromatic regions.

The left of the figure shows the plate that had no drug, the one on the right had hygromycin (hyg) added. The bottom three strains have the *hph* gene inserted. The strains that were able to grow on hyg were expressing the *hph* gene and subsequently had improper heterochromatin formation.

Creating CDP-1 Domain Mutants

In order to test which domains of CDP-1 are required for proper function of CDP-1 at heterochromatin, I first had to create strains with specific mutations in each of the domains. I inserted CDP-1 domain mutations with a 3xflag tag at an exogenous locus[47] into a strain with the endogenous[48] copy of *cdp-1* deleted and CenVIR::*bar*. I

[47] Exogenous locus—I inserted the CDP-1 mutations at a region that is not at the normal *cdp-1* gene, in this case, at the *his-3* locus
[48] Endogenous—the area of the genome where CDP-1 is normally found and produced

inserted 5 different CDP-1 mutants into *N. crassa* strains—wild type *cdp-1* (positive control), CD mutant *cdp-1*, AT hook 1 mutant *cdp-1*, AT hook 2 mutant *cdp-1*, and AT hook 1/2 double mutant *cdp-1*. By inserting wild type *cdp-1* into *cdp-1* deletion strains, I expect to rescue the normal phenotype[49] of *N. crassa* strains when *cdp-1* is present and functioning properly. This demonstrates that the insertion of *cdp-1* back into *N. crassa* strains with the endogenous copy deleted can result in normal CDP-1 function and any defect in strains with mutants inserted may be attributed to the specific mutations in CDP-1 domains. Once I have obtained the proper strains, I can perform spot test silencing assays (qualitative) and qRT-PCR (quantitative) to test the expression of the *bar* gene at CenVI. Since I know that *bar* is expressed at CenVI in *cdp-1* deletion strains, I can make conclusions about the function of each of the *cdp-1* mutant strains based on the ability to re-silence *bar* expression. If *bar* is still expressed (strains can grow on an antibiotic medium) in the mutants, I can conclude that the mutant is required for CDP-1 function in heterochromatic silencing at CenVI.

CDP-1 is required for silencing of NCU16718 at telomere IIIR

In addition to determining if CDP-1 is required for silencing at heterochromatic regions, I wanted to test if CDP-1 affects expression of native genes. My mentor first analyzed RNA-sequencing data (data not shown) that revealed some changes in gene expression when comparing WT strains to *cdp-1* deletion strains. I wanted to analyze this finding further. I selected 6 genes that appeared to be altered the most in a *cdp-1* deletion strain and performed qRT-PCR (Figure 11). After running qRT-PCR, I found

[49] Phenotype—the observable characteristics of an organism based on the genotype (e.g. color, size, shape, function)

that one gene, NCU16718[50] was highly upregulated when *cdp-1* was deleted compared to WT. This gene was expressed at levels about 5000x greater than in WT suggesting that CDP-1 is important for silencing this gene. Upon further investigation, we found that this gene was located in a telomeric region on LG III. Overall this experiment tells us that CDP-1 is required for silencing of this gene, NCU16718, at telIII and that CDP-1 is required for heterochromatic silencing at only certain telomeres (not telVII).

[50] NCU16718—gene coding for a hypothetical protein in *N. crassa*. Purpose and function of this gene is unknown.

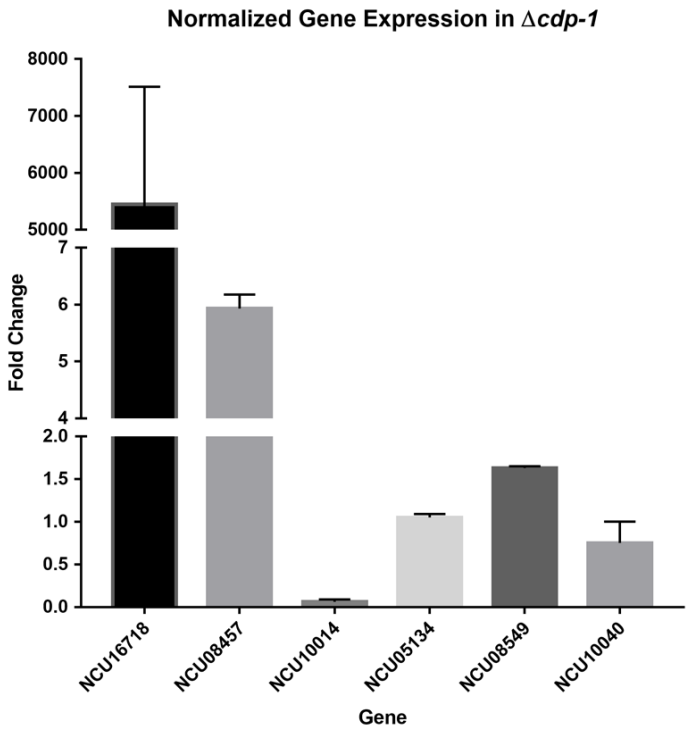

Figure 11: qRT-PCR data for gene expression in cdp-1 deletion strain.

Each gene is normalized to actin (control). A fold change of 1 indicates no change in expression in Δ*cdp-1* compared to wild type. NCU05134 has no significant change in gene expression (~1). NCU10014 (~0) and NCU10040 (~0.8) have decreases in gene expression. NCU08549 has a slight increase in gene expression (~1.5), NCU08457 has a greater increase in gene expression (~6), and NCU16718 has the greatest increase in gene expression (~5000).

CDP-1 interacts with chromatin associated proteins in *N. crassa*

Knowing that CDP-1 has some role at heterochromatin in *N. crassa*, the next step was to determine if CDP-1 associates with other proteins at heterochromatin. Shinji Honda (a former post doc in the Selker lab) performed two large scale pull-down assays with flag-tagged CDP-1. His results suggest that CDP-1 interacts with a handful of proteins in *N. crassa*. My mentor and I decided to focus on four proteins that appeared in both assays, CRF8-1, POB-3, SPT-16, and HIR-1. We focused on these specific

proteins because they have been shown to have roles at chromatin. CRF8-1 is a chromatin remodeler[51], POB-3 and SPT-16 (part of the FACT complex) are histone chaperones[52], and HIR-1 (part of the HIRA complex) is also a histone chaperone. If CDP-1 interacts with these proteins in *N. crassa*, we will have a better understanding of what exactly CDP-1 is doing at heterochromatin.

In order to confirm the interaction between CDP-1 and these potential interacting partners, I performed Co-immunoprecipitation (Co-IP)[53]. First, I inserted tagged constructs[54] of each protein (CRF8-1, POB-3, SPT-16, and HIR-1) into its endogenous locus in separate *N. crassa* strains. CDP-1 (previously tagged) was tagged with 3xflag::hph (CDP-1-3xflag::hph) and the interacting partners were tagged with 3xHA::hph ("protein"-3xHA::hph). I crossed CDP-1 tagged strains and interacting partner tagged strains to obtain strains with both tagged constructs or just HA-tagged protein to use as a negative control. Next, I performed the Co-IP procedure with antibody coated beads[55] specific to the flag tag. By using flag beads, I am binding the CDP-1-3xflag (also referred to as "pulling down"). Later, in a Western blot analysis[56], I used an HA antibody[57] to determine if any of the 3xHA::hph tagged proteins associated with CDP-1 when I pulled CDP-1 down with the flag beads.

[51] Chromatin remodeler—regulatory protein that alters chromatin structure to allow transcriptional proteins access to DNA
[52] Histone chaperones—proteins that guide histones to their proper positions within the nucleosome
[53] See methods.
[54] Tagged constructs—same as epitope tag, see methods
[55] Antibody coated beads—insoluble beads that are coated with antibodies specific to proteins or epitope tags on proteins.
[56] See methods
[57] HA antibody—protein that binds to the HA tag and can help visualized proteins on a Western blot

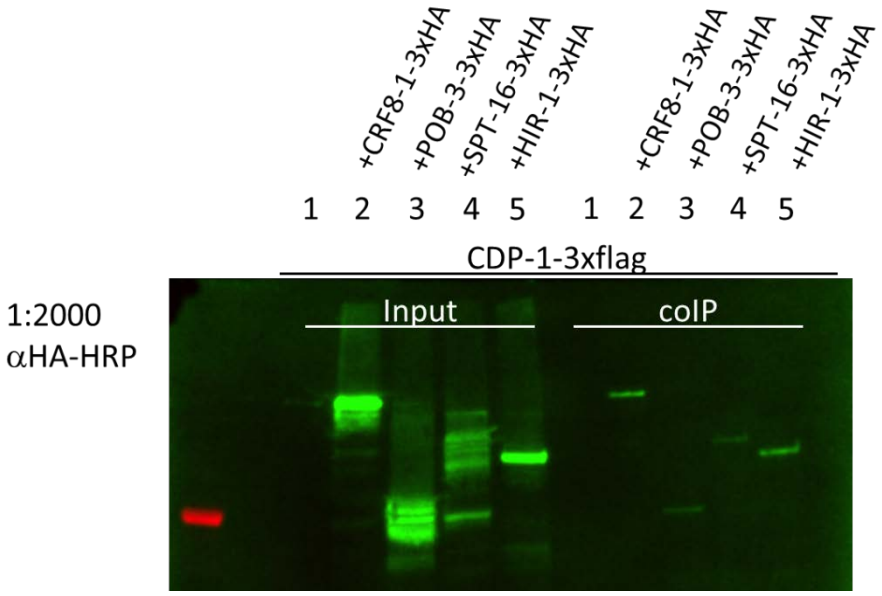

Figure 12: Co-immunoprecipitation.

This Western blot is probed with an HA antibody (αHA), binding to HA-tagged proteins present in strains (input lane 2, 3, 4, 5 and in Co-IP lanes 2, 3, 4, 5 if protein interacts with CDP-1)

Based on my results, CDP-1 does interact with each protein. The first five lanes in Figure 12 show the input of protein before immunoprecipitating with flag beads and the last five lanes show protein after pull-down of CDP-1 with flag beads. Since this blot was probed with HA antibody, the bands are showing the proteins tagged with 3xHA. The first five lanes show that the 3xHA tagged proteins are being expressed in these strains. The last five lanes show that after addition of flag beads, which specifically pulls-down CDP-1-3xflag, 3xHA tagged proteins precipitated with CDP-1. This result demonstrates that CDP-1 interacts with each interacting partner in *N. crassa*.

Discussion

CDP-1 localizes to heterochromatin, via H3K9me, and appears to be limited to centromeres and select telomeres potentially due to a mechanism involving CRF8-1, FACT components, or HIRA components. I have demonstrated that CDP-1 is required for heterochromatic silencing at CenI, in addition to CenVI, suggesting that CDP-1 may have a role at all centromeres (seven) in *N. crassa*. In order to determine if CDP-1 does have a role in heterochromatic silencing at all centromeres, the other centromeres need to be examined. If antibiotic resistance genes were inserted into the remaining centromeres and all showed heterochromatic silencing defects in the absence of *cdp-1*, we can determine that CDP-1 is required for heterochromatic silencing at all centromeres. However, there is a possibility that CDP-1 silencing function is specific to certain centromeres, similar to the telomeres. If this is the case, we would need to investigate the subtleties (e.g. DNA methylation, histone methylation) that help CDP-1 distinguish one centromere from another.

I have revealed that CDP-1 has specificity towards select telomeres in *N. crassa*. Previous research in the Selker lab showed that CDP- 1 is not required for heterochromatic silencing at telVIIL. However, I found CDP-1 significantly regulates the expression of NCU16718 in telIIIR, suggesting that CDP-1 has a role in heterochromatic silencing at telIIIR. This gene is a hypothetical protein in genome databases, does not have any studied homologues in other organisms, and has yet to be tested for any apparent role in *N. crassa* processes. In order to distinguish differences in this region of telIIIR between WT and *cdp-1* deletion strains, I looked at DNA methylation Southerns (data not shown). The DNA methylation Southerns examined

DNA methylation patterns in this region and showed that there were changes in DNA methylation patterns between WT and *cdp-1* deletion strains. However, this finding suggests that a single nucleotide polymorphism (SNP)[58] in NCU16718 may have altered the sequence of a restriction cut site in addition to altering gene expression (rather than CDP-1 altering gene expression); nonetheless, these findings are incomplete. To supplement this finding, we need to sequence NCU16718 in the WT and *cdp-1* deletion strains to determine if there is a SNP overlapping with a restriction cut site. Although this does not explain why deletion of CDP-1 causes upregulation of NCU16718, it demonstrates that a potential mechanism is changing this gene when *cdp-1* is deleted. Further investigation into ChIP-seq data may show details about changes in heterochromatin at this gene.

I also demonstrate that CDP-1 is not required for heterochromatic silencing at certain interspersed heterochromatic regions. There are many interspersed heterochromatic regions dispersed throughout the *N. crassa* genome. Again, to get a better understanding of CDP-1 function we should investigate more interspersed heterochromatic regions to obtain a more definite view of CDP-1 at these regions and confirm that CDP-1 is not required for heterochromatic silencing at interspersed heterochromatic regions.

CDP-1 has 3 distinct domains—a chromodomain and two AT hook domains. In other heterochromatic proteins, the chromodomain allows proteins to recognize and bind to H3K9me and I hypothesized that it plays the same role in CDP-1 and is required for CDP-1 localization to heterochromatin. The AT hooks have also been shown to help

[58] Single nucleotide polymorphism—a type of mutation that changes one nucleotide in a DNA sequence

proteins bind to AT-rich DNA regions and since *N. crassa* heterochromatin is generally AT-rich, CDP-1 AT hooks may also help CDP-1 bind to the AT-rich heterochromatic regions. The CDP-1 mutants of these domains that I constructed may indicate how CDP-1 successfully localizes to heterochromatic regions.

Understanding what CDP-1 does independently at heterochromatin is important to construct a basic picture of CDP-1 function; however, understanding CDP-1 interaction with other proteins at heterochromatin will provide us with a broader, complex picture of CDP-1 role at heterochromatin. I have demonstrated that CDP-1 interacts with other chromatin associated proteins in *N. crassa*. As a supplement to Figure 12, we need to ensure that the 3xflag beads used are specific to CDP-1-3xflag and do not have off target effects (bind to HA tag or potential interacting proteins). Another Co-IP with additional strains that have only HA tagged proteins will confirm if the 3xflag beads are specific. Assuming that the 3xflag beads are specific to flag tagged proteins, we may hypothesize that CDP-1 is working with these complexes which would provide insight into the function of CDP-1 in *N. crassa*.

Based on these findings, I can conclude that CDP-1 is important for heterochromatic silencing and proper heterochromatin formation. However, the silencing function of CDP-1 may be limited to only certain heterochromatic regions, such as CenVI, CenI, and telIII. Overall, CDP-1 does appear to have a function at heterochromatin and may have a role in proper cellular processes, such as mitosis and gene regulation. More research is required to fully understand the function of CDP-1 at *N. crassa* heterochromatin.

Methods

Neurospora crassa strains and DNA isolation

N. crassa strains were grown, cultured, and crossed as previously described [8][9]. DNA isolation procedures were followed from Honda & Selker, 2009 [8]. *N. crassa* strains were grown in Vogel's minimal media with 1.5% sucrose and any necessary supplements, shaking at 32^{o} C for 2 days and then DNA was isolated.

Spot test silencing assay

Modified from Smith KM, et al, 2008, 500,000 cells of conidia from *N. crassa* strains were diluted (using serial dilutions) in 190 μl of sterile water [13]. 2 μl of conidial dilution was "spotted" on FGS medium (with necessary supplements) with or without 1x/2x/3x/4x selective antibiotic medium and grown in 25^{o} C incubator. Plates were analyzed and photographed every 24 hours up to 10 days.

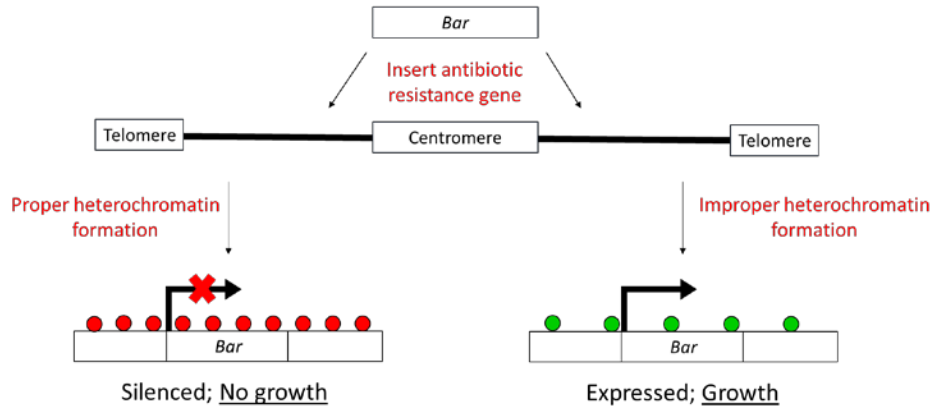

Figure 13: Schematic of spot test silencing assay.

The *bar* antibiotic gene can be replaced with other antibiotic resistance genes (e.g. *nat-1*, *hph*). After the antibiotic resistance gene is inserted, the gene of interest is knocked out.

Gel electrophoresis and Southern blot analysis

DNA was prepared following standard procedures described in Klocko, et al. 2015 [17]. Modified from Miao, et al. 2000, 2 µl of DNA was digested with 1 µl of restriction endonuclease overnight for complete digestion [18]. The digested DNA was run on 0.8% agarose gels in 1x TBE buffer at 180V. For Southern analysis, hybridized probes with 4 µl ^{32}P specific to genomic regions were used.

Construction of CDP-1 mutants for analysis

As previously described, plamids were constructed and targeted to the *his-3* locus in *N. crassa* [8]. Mutants were then crossed to obtain homokaryons. In order to confirm proper insertion of the mutations, Southern blot analysis was used.

qRT-PCR

As described in Klocko, et al. 2015, for qRT-PCR analyses, cDNA samples were prepared from RNA samples [17]. RNA was prepared following procedures from

Roundtree & Selker 1997 [19]. For qRT-PCR analysis, FAST SyBr Green master mix was used with forward and reverse primers for actin, NCU16718, NCU08457, NCU10014, NCU05134, NCU08549, and NCU10040. The raw data was normalized to actin.

Epitope-tagged proteins, protein extraction and Coimmunoprecipitation[59]

Using knock-in methods, CRF8-1, POB-3, SPT-16, and HIR-1 were tagged with 3xHA::hph [8]. Strains were crossed to CDP-1-3xflag::hph strains using standard procedure [9]. Crosses were germinated on 1x hygromycin FGS medium to select for 3xHA::hph tagged proteins as well as CDP-1-3xflag::hph. Epitope-tagged proteins were confirmed through Western blot analysis previously described in Klocko, et al. 2015 using HA and flag antibodies [17]. The Co-IP procedure used was previously described in Honda & Selker 2009 [8].

[59] See references for more detail [11][12]

Bibliography

1. Grewal SIS, Jia S (2007) Heterochromatin Revisited. *Nature Review* **8**: 35-46

2. (2008) Epigenetic Modifications Regulate Gene Expression. SAbiosciences **8**: 2-5

3. Aramayo R, Selker EU (2013) *Neurospora crassa*, a Model System for Epigenetics Research. Cold Spring Harb Perspect Biol **5**: 1-18

4. Pray L (2008) Eukaryotic genome complexity. Nature Education 1(1):96

5. Sullivan BA, Karpen GH (2004) Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. Nature Struc & Mol Bio. **11**, 1076-1083

6. Lewis ZA, Adhvaryu KK, Honda S, Shiver AL, Knip M, et al. (2010) DNA Methylation and Normal Chromosome Behavior in Neurospora Depend on Five Components of a Histone Methyltransferase Complex, DCDC. PLoS Genet 6(11): e1001196. doi:10.1371/journal.pgen.1001196

7. Aravind L, Landsman D. (1998) AT-hook Motifs Identified in a Wide Variety of DNA-binding Proteins. Nucl. Acids Res. 26(19): 4413-4421

8. Honda S, Selker EU. (2009) Tools for Fungal Proteomics: Multifunctional Neurospora Vectors for Gene Replacement, Protein Expression and Protein Purification. Genetics 182(1): 11-23

9. Davis RH (2000) Neurospora: Contributions of a Model Organism. Oxford: Oxford University Press. 333 p.

10. Jamieson K, Wiles ET, McNaught KJ, Sidoli S, Leggett N, Shao Y, Garcia BA, Selker EU (2016) Loss of HP1 Causes Depletion of H3K27me3 from Facultative Heterochromatin and Gain of H3K27me2 at Constitutive Heterochromatin. Genome Res 26(1): 97-107

11. Brizzard B. (2008) Epitope Tagging. Bio Techniques 44: 693-695

12. (2010) Thermo Scientific Pierce: Protein Interaction Technical Handbook Version 2.

13. Smith KM, et al. (2008) The Fungus *Neurospora crassa* Displays Telomeric Silencing Mediated by Multiple Sirtuins and by Methylation of Histone H3 Lysine 9. Epigenetics Chromatin 1(1): 5

14. Lewis ZA, et al. (2009) Relics of Repeat-induced Point Mutation Direct Heterochromatin Formation in *Neurospora crassa*. Genome Res 19(3): 427-437

15. Irelan JT, Selker EU. (1997) Cytosine Methylation Associated with Repeat-Inducted Point Mutation Causes Epigenetic Gene Silencing in *Neurospora crassa*. Genetics 146: 509-523

16. Smith KM, et al. (2010) H2B- and H3-Specific Histone Deacetylases are Required for DNA Methylation in *Neurospora crassa*. Genetics 186(4): 1207-1216

17. Klocko AD, Rountree MR, Grisafi PL, Hays SM, Adhvaryu KK, Selker EU. (2015) Neurospora Importin α is Required for Normal Heterochromatic Formation and DNA Methylation. PLoS Genet. 11(3)

18. Miao VPW, Freitag M, Selker EU. (2000) Short TpA-rich Segments of the ζ–η Region Induce DNA Methylation in *Neurospora crassa*. J. Mol. Bio. 300: 249-273

19. Rountree MR, Selker EU. (1997) DNA Methylation Inhibits Elongation but Not Initiation of Transcription in *Neurospora crassa*. Genes and Dev. 11: 2383-2395

20. Freitag M, Hickey PC, Khlafallah TK, Read ND, Selker EU. (2004) HP1 is Essential for DNA Methylation in *Neurospora*. Mol Cell. 13(3): 427-434

21. Tamaru H, et al. (2003) Trimethylated Lysine 9 of Histone H3 is a Mark for DNA Methylation in *Neurospora crassa*. Nature Genet. 34: 75-79