A MOLECULAR INVESTIGATION OF THE EFFECTS OF THE LOSS OF LINKER HISTONE H1 IN THE FILAMENTOUS FUNGUS NEUROSPORA CRASSA

by

MICHAEL JUSTIN SEYMOUR

(Under the Direction of Zachary Lewis)

ABSTRACT

This study represents an initial investigation into the effects on chromatin structure and gene regulation of *N. crassa* following the deletion of the gene for the linker histone H1 (*hho*). This study concentrated on the use of high throughput sequencing techniques and the analysis of the resulting large data sets generated via mRNA-seq, ChIP-seq, and MNase-seq. Though no differential patterns of H1 occupancy were found, significant changes in the transcription rate of a small set of genes in the H1 deletion (Δ*hho*) strain with accompanying changes in nucleosome stability in the NFR region of these genes indicates a role for H1 in gene regulation and the maintenance of chromatin structure in this organism.

INDEX WORDS: Linker histone H1, *Neurospora crassa*, transcriptional regulation, RNA, high throughput sequencing, microccocal nuclease digestion, chromatin immunoprecipitation, nucleosome

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B.S., Georgia Gwinnett College, 2011

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2014

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August 2014

DEDICATION

This work is dedicated to my family. To my loving wife Tracey, my two beautiful sons, Samuel and Maxwell, and to any children yet to come for giving me the drive and purpose needed to overcome so many obstacles. To my mother Geralyn who instilled in me a love of learning. To my brother Matthew for his steadfast loyalty and support through all the dark places in our lives. In memory of my grandparents, John and Shirley Seymour and Francis and Mildred Coyne, for the gift of their unconditional love and the joys of my childhood. To my in-laws Bruce and Gail, for accepting me into their family, their heartfelt advice, and their innumerable acts of kindness. To Erik, Kathy and Michael Britton for all their love, guidance, and moral support through all these many years. To all those not mentioned here in the past, present, and future: *Non mihi, non tibi, sed nobis*

ACKNOWLEDGEMENTS

I would like to thank Dr. Lewis for his guidance and support over the past three years. I would also like to thank the other members of my committee, Dr. Timothy Hoover and Dr. Xiaoyu Zhang. To all my lab-mates and the many friends I have made during my time here, thank you for sharing the frustrations, the successes, and the laughs.

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CHAPTER 1

INTRODUCTION

Eukaryotic DNA is associated with various chromatin proteins, which include both histone and non-histone proteins. The basic unit of chromatin is the nucleosome, which consists of 146 bp of genomic DNA wrapped approximately twice around a core histone octamer (composed of two copies of each of the following histones: H2A, H2B, H3, H4) [1, 2]. The core histone octamer serves as a spool around which the DNA thread is wrapped. The DNA is then bound by the linker histone H1 as it enters and exits the nucleosome [3-5].

Histone H1, hereafter referred to as H1, is also known as the linker histone. H1 is a highly conserved and widely distributed member of the histone family of proteins [6, 7]. Unlike the core histones, H1 is not contained within the core nucleosome octamer, and there is only one copy of H1 per nucleosome [3-5]. H1 also has a different basic structure in comparison to the core histones [6, 7]. H1 is unique in terms of its binding and interaction with chromatin, and may serve to influence higher order chromatin compaction [3, 8-10]. Though current understanding of the structure and function of the core histone proteins is well advanced, knowledge of H1's function in chromatin regulation and higher order structure is currently limited.

To rectify this deficit, I have initiated an investigation of H1's role in chromatin structure and function by exploring its interactions with, and role in, chromatin structure and regulation in the filamentous fungus *Neurospora crassa*. *N. crassa* is a coenocytic,

heterothallic, haploid organism that has a modest set of nutritional requirements [11]. In addition, unlike most other eukaryotic organisms, *N. crassa* has a single gene for H1 (*hho*) [12, 13]. Over the last century *N. crassa* has been widely used to study different aspects of eukaryotic biology, which has led to many great discoveries in genetics, cellular metabolism, and most recently epigenetics [14, 15]. As a result, a large body of literature and an expansive array of investigative techniques have been developed and optimized for use with *N. crassa*, making it an ideal candidate to serve as a platform for the study of H1.

The work presented in the following sections of this thesis focuses on the use of various molecular biological techniques to investigate the effects of knocking out the gene for H1 (*hho*) in *N. crassa* and comparing it to wild-type strain S1. The techniques focus on the collection of large sequencing data sets to take an in-depth look at changes effected in the transcriptome and changes in nucleosome spacing and occupancy on the genome as a result of this deletion.

CHAPTER 2

LITERATURE REVIEW

Histone H1, also known as the linker histone, is a highly conserved and widely distributed member of the histone family of proteins [6, 7, 16]. Unlike the core histones, H1 is not contained within the core histone octamer, but is present in only one copy per nucleosome [3-5]. H1 also has a different basic structure in comparison to the core histones [6, 7]. Histone H1 is unique in terms of its binding and interaction with chromatin, and may serve to influence higher order chromatin compaction [3, 8, 9]. Though current understanding of the structure and function of the core histone proteins is well advanced, our knowledge of H1's function in chromatin regulation and higher order structure is currently limited. To rectify this deficit, I have begun an investigation into H1's role in chromatin structure and function by exploring its interactions with, and role in, chromatin higher order structure and regulation. However, before the specific problems of H1 can be addressed, it would be helpful to first put it into the context of an epigenetics background.

Early Epigenetics History

The word epigenetics was first coined by the prominent embryologist Conrad Waddington, who broadly defined it as the unfolding of the genetic program for development [17-19]. It was not until much later in the last century that the modern definition of the term came to solidify, mainly around the writings of R. Holliday, who

defined it as the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms [20, 21]. Early studies of epigenetic phenomena included those of B. McClintock who studied "controlling elements" in maize [22], and J. Schultz who studied heterochromatin in Drosophila [23]. Later work by R. A. Brinks on the R locus in maize [24, 25], and M. Lyon's work on X chromosome inactivation in mammals [26], demonstrated the differences between epigenetic and genetic systems. However, no real molecular mechanisms of action were known at the time.

Chromatin Regulation

It was not until 1969 that the first real molecular mechanism was proposed to explain part of chromatin's regulatory control function. DNA methylation was first identified by Griffith and Mahler in 1969 as a possible candidate for a method of epigenetic control of gene expression, though they had no proposed specific molecular model [27]. It was not until 1975 that a molecular model for the control of gene activity through the methylation of cytosine was independently discovered by A. D. Riggs, and the team of R. Holliday and J. E. Pugh [28-30]. These models were based on the molecular activation / inactivation of regions of DNA by a sequence specific methylating enzymes that would function in a heritable fashion. Riggs specifically addressed X chromosome inactivation, while Holliday and Pugh mentioned the possibility of control by molecular clock mechanisms and the possibility of cytosine deamination as discussed in earlier papers by Scarano [31, 32]. These proposals were supported by further papers by R. Sager and R. Kitchin who proposed the enzymatic restriction of unmodified DNA

in eukaryotic organisms [33]. Additional research indicated an important role for DNA methylation in DNA repair, mutation, and recombination in the prokaryote *E. coli* [34-39] and in eukaryotic systems [40, 41].

As focus intensified on DNA methylation as a means of epigenetic inheritance and control, a landmark paper in 1987 by R. Holliday laid the groundwork for the surge in epigenetic research in the 1990s [42]. Holliday argued that some non-Mendelian trans-generational effects may be due to the transmission of DNA methylation patterns (or lack thereof) in reproductive cells. He also coined the term "epimutation" to denote heritable changes that were not attributable to DNA sequence. Though continual discoveries have been made in the modification and regulations of DNA methylation since that time [43, 44], our focus must now turn to the parallel study of chromatin and the so-called Histone Code.

Early Chromatin History

Originally the term chromatin was not associated with epigenetics and long predates it. The term chromatin was first coined by W. Flemming in 1882, shortly after the discovery of nucleic acids based on the microscopic observations of dividing nuclei [45]. However, due to the limits of the microscopes and chemical techniques available at the time, not much more was done with chromatin until the middle of the 20th century. Even before the riddle of DNA's structure was solved in the 1950s, it was known that DNA associated with certain nuclear proteins in eukaryotic organisms [46, 47]. Investigations by D. Manzia and J. Schultz during the late 1930s and early 1940s had shown that nucleo-proteins were essential to chromosome structure [48, 49]. Over the

course of the next few decades, very little progress was made in the study of chromatin structure, though certain basic structural riddles such as the α-helix structure in proteins were solved during this period [50].

Even given these advances, very little was actually known about the role and function of chromatin and the histone proteins. An important pair of studies in the late 1960s discovered that posttranslational modifications of histones affected RNA transcription rates, but as yet no comprehensive model of chromatin structure had been proposed [51, 52]. It was not until the early 1970s that the techniques of low angle x-ray diffraction and electron microscopy were finally turned to the study of chromatin structure, leading to the first major model of chromatin structure, the super-helix [53-55]. While the initial super-helical model proved incorrect, it paved the way for the discovery of the nucleosome by A. Olins, D. Olins, and C. Woodcock in 1973 and its characterization as the basic unit of chromatin structure [1, 2, 56-59].

Chromatin Structure

Since the 1970s our knowledge of chromatin structure has grown and a standard model of chromatin's basic unit, the nucleosome, has been refined and verified experimentally. The current model states that the DNA of all eukaryotic organisms associates with various chromatin proteins, which include both histone and non-histone proteins, resulting in the complex structure known as chromatin. The nucleosome is now known to consist of 146 bp of genomic DNA wrapped approximately twice around a core histone octamer. This histone octamer is composed of two copies each of the core histones H2A, H2B, H3, and H4 [1, 2, 58, 60].

The core histone proteins have a common central structure known as the histone fold, which consists of a long central helix bordered on both ends by a helix-strand-helix motif [57, 61]. Heterodimers of histone H3 and H4 form from their respective monomers due to hydrophobic interactions in a head to tail arrangement. Two of these H3 / H4 heterodimer subunits then further interact to form a tetramer consisting of two H3 and two H4 histone proteins. H2A and H2B interact in a similar fashion to form heterodimers, which are subsequently bound to either end of the histone H3 / H4 tetramer to complete the assembly of the histone octamer [1, 2, 8, 57, 58, 60-62].

This octamer is then semi-symmetrically wrapped by ~146bp of genomic DNA which completes approximately 1.6 full turns around histone octamer. This is akin to a thread (the DNA) wrapping around a spool (the histone octamer) to form the core nucleosome particle. The further binding of this complex by the linker histone H1 facilitates the compaction of higher order chromatin structures. Histone H1 binds the DNA as it enters and exits the nucleosome core particle to form a chromatosome [1, 3-5, 8, 61, 63].

Further compaction of chromatin through nucleosome interactions, histone modifications, and the association of other chromatin binding and modifying proteins have been shown to play an important role in gene regulation through the creation and maintenance of euchromatic (transcriptionally active) and heterochromatic (transcriptionally silent) chromatin domains [64, 65]. In most organisms these chromatin regions are marked by distinct patterns of histone Post-Translational Modifications (PTMs) [66, 67]. One widespread PTM is the methylation of histone N-terminal tails, though other PTMs such as acetylation and phosphorylation are also commonly seen [68,

69]. The resulting pattern of epigenetic marks is often referred to as the Histone Code, which is believed to serve as an additional layer of regulatory information above and beyond that encoded in the DNA [70]. The functions of the core histones and their modifications have become increasingly well understood over the last few decades. In contrast, the detailed functions of H1 are comparatively unknown and warrant further investigation [71, 72]. This thesis project initiates an investigation into the effects of deletion of the single H1 gene (*hho*) in the model organism *N. crassa*.

History of *Neurospora crassa*

Neurospora crassa is a filamentous fungus of the phylum Ascomycota (Ascomycetes) that was first noticed as a bread mold infestation in French bakeries in the 1840s [73], and is characterized by an easily recognizable asexual stage with bright orange asexual spores (conidia) [74]. Neurospora species are found throughout most tropical and subtropical areas of the world, as well as many temperate zones, especially in conjunction with agriculture and commerce. In the wild it is often one of the first colonizing species in areas of burned vegetation [75]. *N. crassa* is a coenocytic, heterothallic, haploid for most of its lifecycle, and has a modest set of nutritional requirements that have made it an ideal model organism for the study of eukaryotic biology over the course of the last century [11]. Study of this organism has led to many great discoveries in genetics, cellular metabolism, and most recently epigenetics [14, 15]. As a result, a large body of literature and an expansive array of investigative techniques have been developed and optimized for use with this organism, making it an ideal candidate to serve as a platform for the study of H1.

The first mention of the genus *Neurospora* in a scientific context began with the work of B. Dodge and C. Shear in 1927 though the species had been previously described under other names [73]. During the process of their investigation of bread mold fungi, Dodge and Shear christened the genus *Neurospora* and carefully reclassified the old species known as *Monila sitophila* into four new species under the names *N. sitophila*, *N. crassa*, *N. tetrasperma*, and *N. erythraea*. Their work described the characteristics of the genus' four species and, through the careful analysis of phenotype, mating patterns, and the tetrad analysis of ascospores, conclusively showed *N. crassa* to be a heterothallic species. This represents the first use of Neurospora in the context of genetic analysis, and resulted in the discovery of its two mating types and their perfect Mendelian 4:4 segregation pattern in the progeny [73].

This work was then built upon by C. C. Lindegren, who proceeded to establish the first detailed genetic maps of the species, characterized multiple mutant phenotypes, and helped to establish *N. crassa* as the textbook example of the segregation of alleles during meiosis in haploid organisms [76-78]. This work in turn led to that of G. Beadle and E. Tatum, who used Neurospora as their model for the exploration of the one gene-one enzyme hypothesis. They started by establishing a new methodology for the generation of mutants, ultimately leading to their famous paper in 1945, which supported the validity of the one gene-one enzyme hypothesis and established the link between genes and biochemical reactions [74, 79, 80]. This landmark work and its follow up investigations by N. H. Horowitz and U. Leupold [81-85] effectively started molecular biology and biochemical genetics in their modern forms. By clearly demonstrating the genetic foundation of cellular metabolism, and by establishing a versatile and thorough

methodology, this group of researchers opened the door for such investigations in other key model organisms such as the now ubiquitous *Escherichia coli* [86].

After the advent of the age of *E. coli*, Neurospora research shifted focus to those issues specifically related to eukaryotic and fungal biology, yielding many critical discoveries. Its versatility as a model organism was amply demonstrated over the last half of the 20th century with a long list of advances in our understanding of fungi, and eukaryotes in general. Important discoveries were made in translational and metabolic suppression [87-89], complementation [90, 91], coordinated control of unlinked genes [92-94], membrane transport mechanisms [95-100], mitochondrial descent and regulation [101-106], circadian rhythms and clock regulation `[107-116], vegetative incompatibility [117-119], and gene conversion [120, 121], to name a few.

More recently Neurospora has become one of the premier models for investigation into the epigenetic regulation of the eukaryotic genome. Neurospora has many advantages for use as a model system in epigenetic research, not the least of which is that its genome has been comprehensively sequenced, mapped and annotated [122], and that a comprehensive knockout library is available through the fungal genomics stock center [123-125]. Neurospora has been successfully used to elucidate complex epigenetic mechanisms such as Repeat Induced Methylation (RIP) [126-128], Meiotic Silencing by Unpaired DNA (MSUD) [129, 130], and Quelling [131, 132]. Additionally, many other elements of epigenetic control have been worked out in the organism, including research into the nature of centromeric DNA and repeats [133], the characterization of chromosome ends [134], and research into both DNA and histone methylation as a means of epigenetic control [135-140]. In light of these advances in the understanding of the

epigenetic environment of *N. crassa*, I believe that this organism will serve as an excellent model to use in this project.

Histone H1 Gene Family and Chromatin Structure

H1 is present in most eukaryotic cells studied to date [141-143], and in most organisms, the structure of H1 is highly conserved [6, 7]. Unfortunately, it has been difficult to determine the in vivo functions of H1 for a number of reasons. H1 appears to be essential for viability in metazoans, and the existence of multiple H1 variants complicates in vivo analysis of these proteins in higher eukaryotes [144, 145]. For example, humans encode 11 known H1 variants, consisting of eight genetic and three splicing variants [145]. Some common microbial model organisms such as the budding yeast *Saccharomyces cerevisiae* and the protozoan *Tetrahymena thermophila* possess single H1 homologs, however, these organisms encode H1 proteins with non-canonical structures [141, 146-148]. Other potential eukaryotic model organisms like *Mus musculus* and *Arabidopsis thaliana* possesses canonical H1 proteins, but contain multiple genes and splicing variants that could overcomplicate analysis of H1 interactions [149-154].

In contrast, *N. crassa* has only a single H1 gene (*hho*) with no known splicing variants and has been shown to be dispensable for viability [13, 155]. It is also important to note that the H1 protein found in *N. crassa* is predicted to match the standard canonical H1 structure with an N-terminal tail, central winged helix globular domain, and a long positively charged C-terminal domain [12, 156]. These unique features will allow for valid loss-of-function studies on H1 to be conducted in vivo that may serve to identify unknown interactions or mechanisms of H1 regulation and function. Furthermore, as

established above, *N. crassa* is a well-established model system with a diverse array of available resources [157, 158]. Although H1 has been difficult to study, it is thought to play an important role in chromatin compaction [159], and some evidence exists for its role in transcriptional regulation [9, 12, 160, 161]. However, much of what is known about H1's structural role stems from early studies conducted in vitro [162-164]. H1's exact functions in vivo are still not completely understood and the ability to study the effects of its absence in *N. crassa* should prove extremely valuable [145, 150].

In addition to existing in multiple copies and variants, previous studies have shown that H1 can undergo a variety of PTMs in a manner similar to the core histones [145, 165, 166]. Though many of these PTMs have no known function, they may serve to alter H1's chromatin-binding affinity or its interaction with other chromatin binding proteins, in a manner similar to the PTMs of the core histone proteins [66, 141, 145]. H1 has previously been demonstrated to bind dynamically to chromatin, jumping on and off nucleosomes with a residence time ranging from 45 seconds to as much as 3 minutes [167, 168]. There is also evidence H1 competes with other non-histone chromatin proteins, such as High Mobility Group (HMG) proteins and PARP-1, for chromatin residency [169-171]. PTMs of H1 could serve to inhibit or enhance these binding and competitive functions.

The exact mechanisms of histone H1's interaction with the core histone proteins and its method of interaction with linker DNA are also inadequately defined, as is its role in higher order chromatin compaction [159, 172-174]. Previous studies indicate that H1 may initially bind to the linker DNA between nucleosomes through an ionic interaction between H1's positively charged C-terminal tail and the negatively charged linker DNA

[175, 176]. Though H1 has been shown to induce compaction in isolated chromatin *in vitro* [162], its exact role in chromatin compaction and structural regulation in vivo is not fully understood [150, 177]. There is also relatively little known about H1's specific protein-protein interactions within the cell. The core histones are known to interact with chaperone proteins [178-180], PTM enzymes [66, 181], chromatin remodelers [182], and other chromatin binding proteins [183], however, H1's role in these types of interactions are not yet fully understood [184, 185].

Objective of Thesis

The work presented in the following sections of this paper will focus on establishing *N. crassa* as a strong model organism for the investigation of histone H1 that will provide insights into the function and role of H1 in the regulation of chromatin in higher eukaryotic organisms [186]. Experiments will focus on studying the effects of the lost of the H1 gene (*hho*) in *N. crassa* through the collection of large sequencing data sets via a variety of molecular biological techniques. This should serve to provide a baseline of knowledge about H1's possible metabolic and structural roles that can serve as a firm foundation for later more detailed experiments in *N. crassa*.

CHAPTER 3

MATERIALS AND METHODS

Strains and Growth Conditions

Wild-type strain S1 (FGSC #4200) and strain *Δhho* (Δ Histone H1 mutant) (FGSC #12224) were obtained from the Fungal Genomics Stock Center Collection [123-125]. Lab Strain LX-44-5 (3x FLAG tagged *hho*) was developed using established techniques by Dr. Lewis (Lewis Unpublished) [157].

Neurospora crassa cultures were grown in an incubator at 32°C in solid or liquid Vogel's minimal medium (VMM) with 2% sucrose according to established procedure unless otherwise noted [187]. Liquid cultures were shaken at 150 rpm during growth, while solid culture media were not agitated but were amended with the addition of 1% agar before inoculation unless otherwise noted [187].

Preparation of conidial suspensions was done according to established procedure unless otherwise noted [187]. 25 ml of sterile de-ionized filtered water was added to the desired culture grown on solid media (see above) after at least 6 days of growth with a minimum of 24 hour light exposure. Cultures were then vortexed vigorously to suspend conidia. The resulting supernatant was then filtered through sterile cheesecloth to remove loose agar and mycelia. The resulting filtrate was then poured into a 50 ml Falcon Tube and centrifuged @ 3000 rpm for 10 minutes in swinging bucket centrifuge. The supernatant was then carefully pipetted off and discarded. Conidia were then re-

suspended by bringing volume up to 5 ml with sterile filtered de-ionized water, vortexed, and conidial concentration calculated through the use of a hemocytometer.

ChIP Sample Preparation

ChIP-seq experiments were conducted to determine H1's occupancy on the genome of *N. crassa*. Seven day stock cultures of strain LX-44-5 (3x FLAG tagged *hho*, see above) were grown on solid media at 32°C and allowed to conidiate in sunlight for 24 hours, after which a conidial suspension was prepared according to established procedures (see above). Conidial suspensions were used to inoculate 50 ml of liquid media (2% Glucose, 1X Vogel's salts), with $5x10^6$ conidia per milliliter of media inoculated. Samples were allowed to grow for 5 hours at 32°C at 180 rpm. After incubation germinated conidia (germlings) were centrifuged at 3000 rpm for 10 minutes to collect cells and the supernatant discarded. Germlings were washed once with 40 ml of 1X PBS, centrifuged at 3000 rpm for 10 minutes and the supernatant discarded. Germlings were then re-suspended in 10 ml of 1X PBS and chemically cross-linked with formaldehyde (1% final concentration) on a rotating platform for 30 minutes at room temperature, after which the reaction was quenched with glycine (125 mM final concentration). Conidia were then washed twice with 40 ml of 1X PBS, collected by centrifugation as above, and re-suspended in 1 ml of ice cold ChIP lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 0.1% deoxycholate) with protease inhibitors (final concentrations of 0.1 mM PMSF, 1X Pepstatin, and 1X Leupeptin).

The resulting samples were then split into two equal aliquots in 1.5 ml epi-tubes and placed on ice. Chromatin was mechanically sheared by sonication using an Ultra Sonic processor (Heat System-Ultrasonics Inc; 80 duty cycle, 3.5 output) to deliver 6 sets of pulses (30 pulses, 1second duration per pulse). Samples were allowed to rest on ice for at least 2 minutes between each pulse set. The resulting lysates were then centrifuged at 14,000 rpm for 5 minutes. The supernatants of each split sample were then carefully pipetted off and recombined into a single sample. From this re-combined sample a 20 µl aliquot was pipetted off and saved at -20°C in a 1.5 ml epi-tube for later use (input sample). The combined samples were then pipetted into a number of aliquots equal to one more than the number of antibodies to be used (# of AB +1 negative control) in 1.5 ml epi-tubes. Each aliquot then had 20 µl of equilibrated protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Inc., CA Cat #SC-2003) and its relevant antibody added as required. For the H1-ChIP experiments 1 µl of α-H1 antibody (Sigma Aldrich, MO Cat #F3165) was added, for H3-ChIP experiments 1 µl of α-H3 antibody (Abcam, MA Cat #AB1791) was added, and for Pol II-ChIP experiments 1 µl of α-Pol II antibody (Covance, NJ Cat #MMS-126R) was added. Samples were then incubated overnight at 4°C with rotation.

After overnight incubation samples were centrifuged at 5000 rpm for one minute, and the supernatant carefully pipetted off and discarded. Samples then underwent a series of ice cold washes. Samples were washed twice with 1 ml of ChIP lysis buffer w/o protease inhibitors (50mM HEPES pH 7.5, 140mM NaCl, 1mM EDTA, 1% triton X-100, 1% deoxycholate), once with 1 ml of ChIP lysis buffer containing 500 mM NaCl, once with 1 ml of LiCl wash buffer (50 mM Tris-HCl pH8.0, 250 mM LiCl, 0.1% IGEPAL

CA-630, and 0.1% deoxycholate), and finally with 1 ml of TE buffer (10 mM Tris- HCl, 1 mM EDTA). After each wash step samples were centrifuged at 5000 rpm for one minute, and the supernatant carefully pipetted off and discarded. After the final wash, bound chromatin was then eluted in 62.5 µl of TES (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 10 minutes, centrifuged at 5000 rpm for 1 minute, and the supernatant pipetted off and saved in a new 1.5 ml epi-tube (repeated once for a total combined elution volume of 125 µl). Eluted chromatin samples were then de-cross-linked overnight at 65°C.

After overnight de-cross-linking samples were brought to a total volume 250 µl with sterile water and treated with 2.5 µl of 10 mg/ml ribonuclease A (Fisher Scientific, MA Cat #BP2539-250) for 2 hours at 50°C. Samples were then treated with 6.25 µl of 20mg/ml proteinase K (Fisher Scientific, MA Cat #BP1700-100) for 2 hours at 50°C. DNA was then extracted by adding 250 µl of phenol/chloroform/IAA (25:24:1) after which the samples were vigorously vortexed. Samples were then centrifuged for 10 minutes at 14,000 rpm and the aqueous portion carefully pipetted off and placed in a new 1.5 ml epi-tube. To the isolated aqueous samples was added 250 µl of pure chloroform and the samples were vigorously vortexed. Samples were then centrifuged for 10 minutes at 14,000 rpm and the aqueous portion carefully pipetted off and placed in a new 1.5 ml epi-tube. Each sample then had 1 µl glycogen (Ambion, MA Cat #AM9510), 32.5 µl of 3M sodium acetate pH 5.2, and 1124.5 µl of 100% EtOH added after which they were allowed to precipitate overnight in a -20°C freezer.

Following overnight precipitation, samples were centrifuged for 5 minutes at 14,000 rpm and the supernatant carefully pipetted off and discarded. Samples were then

washed with 300 µl of 70% EtOH and centrifuged for 5 minutes at 14,000 rpm, after which the supernatant was carefully pipetted off and discarded. Washed samples were dried under vacuum and re-suspended in 25 µl TE buffer and stored at -20°C until needed for library preparation [187, 188].

mRNA Sample Preparation

RNA-Seq was performed to investigate transcriptional changes between the S-1 (Wild Type) and *Δhho* (see above) strains. To accomplish this, 7 day stock cultures of these strains were grown on solid media at 32°C and allowed to conidiate for 24 hours in sunlight, after which a conidial suspension was prepared according to established procedures (see above) [187, 188]. Conidial suspensions were then used to inoculate 500 ml liquid media (2% Glucose, 1X Vogel's salts or 2% EtOH, 2% glucose, 1X Vogel's salts) with $5x10^6$ conidia per milliliter of media inoculated. Samples were then allowed to grow for 6 hours at 32°C at 180 rpm.

After growth cultures were removed and mycelia isolated through the use of a Buchner funnel and #1 filter paper under vacuum. Cultures were slowly poured onto fresh filter paper and allowed to dry, after which the resulting dried mycelia mat was removed with sterile tweezers and immediately placed in liquid nitrogen to flash freeze. The Buchner funnel was wiped down with sterile H_2O followed by 95% EtOH after each replicate. After flash freezing, the mycelial mat was ground to a fine powder in liquid nitrogen using a mortar and pestle, taking care not to allow all the nitrogen to boil off completely. Ground samples were then poured into sterile 50 ml falcon tubes cooled in liquid nitrogen. The nitrogen in the samples was then allowed to slowly boil off and

while the tubes remained immersed in liquid nitrogen, after which the tubes were capped and placed in the -80°C freezer until needed.

To prepare total RNA, ground mycelia samples were retrieved from the -80°C freezer and immediately placed in a bath of liquid nitrogen on the bench to prevent thawing. All of the following reagents were prepared with RNase free components and all equipment and consumables were unopened from distributor or heat treated and sealed until use to prevent RNase contamination unless otherwise noted. New sterile RNase free 1.5 ml epi-tubes were pre-loaded with 500 µl of lysis buffer (final concentrations of 0.1M NaOAc, 1mM EDTA, 4% sodium dodecyl sulfate in RNase free water), and using a sterile RNase free spatula cooled in liquid nitrogen, small aliquots from the appropriate samples of frozen mycelial powder were loaded into each tube, one powdered sample per epi-tube. All samples were then vortexed vigorously and 500 µl of acid phenol/chloroform/IAA (125:24:1) pH 4.5 (Ambion, MA Cat #AM9720) was added while in the fume hood. Samples were again vortexed vigorously after which they were centrifuged for 5 minutes at 14,000 rpm to pellet cell debris. The aqueous portion of the samples were then carefully pipetted off and transferred to new RNase free 1.5 ml epi-tubes. This acid phenol/ chloroform/ IAA extraction was repeated an additional 3 times for a total of 4 washes. After all 4 washes were complete the final aqueous samples had 750 µl of 100% EtOH and 30 µl of Sodium Acetate (pH 5.2) added. The samples were then vortexed vigorously and placed in the -20°C freezer to precipitate overnight.

Precipitated samples were centrifuged for 5 minutes at 14,000 rpm and the supernatant carefully pipetted off and discarded. Samples were then washed with 300 µl of 70% EtOH and centrifuged for 5 minutes at 14,000 rpm after which the supernatant

was carefully pipetted off and discarded. Washed samples were then dried under vacuum and re-suspended in 50 µl of RNase Free water and stored at -20°C [189, 190] until needed. From the total RNA sample, mRNA was isolated with a Qiagen Oligotex® poly A+ isolation kit (Qiagen, MD Cat #70022). The resulting mRNA samples were then stored at -20°C until needed for library construction.

MNase Sample Preparation

MNASE-seq was performed to investigate nucleosome occupancy of the genome in the S1 (Wild Type) and *Δhho* (see above) strains. To accomplish this, 7 day stock cultures were grown on solid media at 32°C and allowed to conidiate in sunlight for 24 hours, after which conidial suspensions for strains S-1 and *Δhho* were prepared according to established procedures (see above) [187, 188]. Conidial suspensions were then used to inoculate 50 ml liquid cultures (2% glucose, 1x Vogel's salts) with $5x10^6$ conidia per milliliter of media inoculated. Samples were then allowed to germinate for 5 hours in a 32°C shaker at 200 rpm. After incubation germinated conidia (germlings) were centrifuged at 3000 rpm for 10 minutes to collect cells and the supernatant discarded. Germlings were washed once with 40 ml of 1X PBS, centrifuged at 3000 rpm for 10 minutes and the supernatant discarded. Germlings were then re-suspended in 10 ml of 1X PBS and chemically cross linked with formaldehyde (1% final concentration) on a rotating platform for 30 minutes at room temperature after which the reaction was quenched with glycine (125 mM final concentration). Conidia were then washed twice with 40 ml of 1X PBS, collected by centrifugation as above, and re-suspended in 1 ml of ice cold NPS buffer with $CaCl_2$ (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 5 mM

$MgCl_2$, 1 mM $CaCl_2$, 1% Triton-X 100, 0.1% deoxycholate, 0.5 mM Spermidine) with protease inhibitors (final concentrations of 0.1 mM PMSF, 1X Pepstatin (Sigma Aldrich, MO Cat #P5318) and 1X Leupeptin (Sigma Aldrich, MO Cat #L9783)).

The resulting Lysates were then centrifuged at 14,000 rpm for 5 minutes. The supernatants of each split sample were carefully pipetted off and discarded and the cell pellets re-suspended in 500 µl of NPS buffer with $CaCl_2$ and protease inhibitors (see above). The re-suspended samples were then transferred to a new 15 mL Falcon tube and brought to 6 ml volume with additional NPS buffer with $CaCl_2$ and protease inhibitors (see above) and placed on ice. The samples were then pipetted into 8 equal 700 µl aliquots in 1.5 ml epi-tubes and placed on ice. Aliquoted samples were then treated with 0.1 µl of Takara Micrococcal Nuclease (Takara, CA Cat #2910A) and placed in 37°C incubator at 200 rpm for varying time intervals (0, 1, 5, 10, 20, 40, 60, 90 and 120 minutes). MNase reactions were stopped by the addition of EDTA and NaCl (final concentration of 10mM and 125mM respectively) after which the samples were de-cross-linked by overnight incubation at 65°C.

After overnight de-cros- linking samples were treated with the addition of 2.5 µl of 10 mg/ml ribonuclease A (Fisher Scientific, MA Cat # BP2539-250) and incubated at 50°C for 2 hours. Samples were then treated with the addition of 6.25 µl 20 mg/ml proteinase K (Fisher Scientific, MA Cat #BP1700-100) and 10 µl of 10% SDS and incubated at 65°C for 2 hours. DNA was then extracted by adding 650 µl of phenol/chloroform/IAA (25:24:1) after which the samples were vigorously vortexed. Samples were then centrifuged for 10 minutes at 14,000 rpm and the aqueous portion carefully pipetted off and placed in a new 1.5 ml epi-tube. To each isolated aqueous

sample was then added 650 µl of pure chloroform, and the samples were vigorously vortexed. Samples were centrifuged for 10 minutes at 14,000 rpm and the aqueous portion carefully pipetted off and placed in new 1.5 ml epi-tubes. Samples were split into two equal volumes in new 1.5 ml epi-tubes, and 1 µl glycogen (Ambion, MA Cat #AM9510), 32.5 µl 3M Sodium Acetate pH 5.2, and 1124.5 µl of 100% EtOH was added to each half sample after which they were allowed to precipitate overnight in a -20°C freezer.

After overnight precipitation samples were centrifuged for 5 minutes at 14,000 rpm and the supernatant carefully pipetted off and discarded. Samples were then washed with 300 µl of 70% EtOH and centrifuged for 5 minutes at 14,000 rpm after which the supernatant was carefully pipetted off and discarded. Washed samples were dried under vacuum and re-suspended in 25 µl TE buffer and stored at -20°C until needed for library preparation [11, 191, 192].

Before sequencing, the digestion of samples were visualized on a 2% agarose gel. Gel bands corresponding to single and double nucleosome fragments were isolated and purified using a Qiagen QIAquick Gel Extraction Kit (Qiagen, MD Cat #28704). Purified samples were then stored at -20°C until needed for library preparation.

Library Preparation and Sequencing

RNA libraries were created using the mRNA samples prepared as detailed above. Libraries were prepared using the Illumina TruSeq® RNA sequencing kit (Illumina: San Diego, CA). Final pooled library samples were sent to the University of Missouri Sequencing Core and sequenced on an Illumina HiSeq 2000.

DNA libraries were created using the ChIP and MNase samples prepared as detailed above. Libraries were prepared using the Illumina TruSeq® DNA sequencing kit (Illumina: San Diego, CA Cat #FC-121-2002). For ChIP samples genomic DNA adaptors were diluted 1:100 before use in library creation. Final pooled library samples were sent to the Oregon State University CGRB Core Laboratories Sequencing Facility for sequencing on an Illumina HiSeq 2000.

Real Time Quantitative PCR

Sequencing results for the H1-ChIP samples were confirmed by qPCR according to established procedures [188]. Primers used in qPCR are listed in Table 1. PCR samples were prepared with iTaq Universal SYBR Green Supermix (Bio-Rad, CA Cat # 172-5121) and 1 µl of diluted ChIP DNA (1:100) on a Bio-Rad iCycler IQ platform (Bio-Rad, CA). Statistical Analyses were performed in Microsoft Excel.

Table 1. Detailed PCR primer table. This table shows the primers used for qPCR confirmation of the H1 ChIP-seq data sets. Primers are listed by gene locus targeted and its linkage group. Additional details such as amplicon size, melt temp, and percent GC content are also provided.

Gene Locus	**Contig**	**Primer Set**	**Amplicon Size**	**Gene Region**	**5' Primer**	**GC content**	**Melt Temp °C**	**3' Primer**	**GC Content**	**Melt Temp °C**
NCU00554	1									
up in *Δhho*		NCU00554-2	92bp	5'UTR	ACACGAGTGCATCACAGCTC	55.0%	60.1	TGCCTCTCACAGATGAGTCG	55.0%	60.1
		NCU00554-3	108bp	CDS	AATGTCGTGCCCTTCATTTC	45.0%	59.9	CTGCTTCTGGTCCTCAAAGG	55.0%	60.0
		NCU00554-5	148bp	3'UTR	CCCTCGCTACTGACCCATAA	55.0%	60.1	ACTGCGAATTTCCATGTTCC	45.0%	59.9
NCU09909	1									
down in *hho*		NCU09909-2	123bp	5'UTR	GAAAGACTCGAACCCCATGA	50.0%	60.0	CCGTTAACTTCCCCACTTCA	50.0%	60.0
		NCU09909-4	96bp	CDS	AGAGGGAGATGGAGGAGGAG	60.0%	59.8	AGAGGATCAGGAGGGCTAGG	60.0%	59.8
		NCU09909-5	110bp	3' UTR	CGCCTCTCCTTCCTAGAGGT	60.0%	60.0	AGTCCAAATGGAAAGCATGG	45.0%	59.9
NCU03561	5									
up in *Δhho*		NCU03651-1	121bp	5'UTR	TTGAAGGACGAGAGGTTGCT	55.0%	59.0	TGAGCGTTTTCAGCATATGG		

Gene Locus	**Contig**	**Primer Set**	**Amplicon Size**	**Gene Region**	**5' Primer**	**GC content**	**Melt Temp °C**	**3' Primer**	**GC Content**	**Melt Temp °C**
		NCU03651-3	141bp	CDS	GCAAGTTCGTCTCCCTCTTC	55.0%	59.0	CAAAGGCGTTCTCAAAGTCC	50.0%	59.0
		NCU03651-6	134bp	3'UTR	GTGCAGCAGCACATTGATCT	50.0%	60.0	CCAAACATTGGAGGGGAGTA	50.0%	59.8
NCU05948	6									
up in *Δhho*		NCU05948-1	108bp	5'UTR	TCTAGGGACCAGGGATGATG	55.0%	59.9	ATGCGGTTATCCTTCGATCA	55.0%	60.4
		NCU05948-4	122bp	CDS	ACTACGCAAGATGGCAGAGG	55.0%	60.4	CCCTCCACAGCTCTTGAATC	55.0%	59.8
		NCU05948-6	108bp	3'UTR	CAGAGCCACCAAACTTCACA	50.0%	59.9	GCAGGCAACTAGGCAGTTCT	55.0%	59.6
NCU02654	1									
down in *Δhho*		NCU02654-1	148bp	5'UTR	ACAACTTGTTCCCCAAGACG	50.0%	60.0	TGTGTGTTGGGGAGGTGATA	50.0%	59.8
		NCU02654-4	120bp	CDS	TCTTCATCCCCGAGTTCATC	50.0%	60.0	CAGACTCGGCTTGGAGAGAC	60.0%	60.1
		NCU02654-5	116bp	3'UTR	TCAGCCTGTCTCAGAAAGCA	50.0%	59.9	GGCTCGGTAGTCAACGGTAA	55.0%	60.1

Gene Locus	**Contig**	**Primer Set**	**Amplicon Size**	**Gene Region**	**5' Primer**	**GC content**	**Melt Temp °C**	**3' Primer**	**GC Content**	**Melt Temp °C**
NCU08739	2									
down in *Δhho*		NCU08739-1	127bp	5'UTR	CCCTGCAACTTCGAACAGAT	50.0%	60.3	TGGCTCGATGGATAGGTAGG	55.0%	60.1
		NCU08739-3	150bp	CDS	TCTGGGTCTTCTCCAACTCC	55.0%	59.2	GATAGACCTGGCCCTTGGAT	55.0%	60.3
		NCU08739-5	123bp	3'UTR	GAAAAGCCGTAGGAGCCTCT	55.0%	60.0	GCAGGAACAGACAAGGAAGG	55.0%	59.8
NCU04173	5									
Tubulin		Tubulin-1	119bp	CDS	ACTACGCCCGTGGTCACTAT	50.0%	60.2	AGGACTACGCCAAGAAGTGC	60.0%	60.7
Control		Tubulin-2	110bp	CDS	TCCATGTTGTCCAACACCAC	50.0%	60.3	ATACCCTCACCGACGTACCA	55.0%	60.3

Data Analysis

ChIP-seq: Data sets were first mapped to the reference genome [13] and were then analyzed for differences in read levels across the genome using the Tuxedo suite of sequencing analysis programs [193]. These programs were utilized through the Galaxy web interface and servers [194-196]. Subsequent analysis was performed by visual inspection of elements with the IGV viewer [197, 198], and pre-processing of data was done in Perl for use in statistical analysis with R (Alexander Matte Santos, Unpublished) [199].

RNA-seq: Data sets were first mapped to the reference genome [13] and were then analyzed for differences in transcript levels across the genome using the Tuxedo suite of sequencing analysis programs [193]. These programs were utilized through the Galaxy web interface and servers [194-196], and preliminary results were stringently screened to minimize the chance of false positives. To do this, transcriptional differences among samples were normalized and a comparison done by FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and log 2 values. In addition, only those genes with at least a log 2 or greater difference in transcript expression levels were accepted as positive hits. Gene transcript functional categories were assigned according to the annotations found on the *N. crassa* database [13]. Subsequent analysis was performed by visual inspection of elements with the IGV viewer [197, 198], pre-processing of data was done in perl for use in statistical analysis with R (Alexander Matte Santos, Unpublished) [199].

MNase-seq: Data sets were first mapped to the reference genome [13] and were then analyzed for differences in read levels across the genome using the Tuxedo suite of

sequencing analysis programs [193]. These programs were utilized through the Galaxy web interface and servers [194-196]. Subsequent analysis was performed by visual inspection of elements with the IGV viewer [197, 198], pre-processing of data was done in perl for use in statistical analysis with R (Alexander Matte Santos, Unpublished) [199].

CHAPTER 4

RESULTS

RNA-Seq

Deletion of H1 results in the mis-regulation of a small subset of genes in *N. crassa*. Histone H1 is believed to play a direct role in the regulation of some genes in eukaryotic organisms. This is supported by previous studies that have examined the effects of H1 depletion in the cell, and found small subsets of mis-regulated genes [150, 151, 200]. H1 has also previously been reported to specifically regulate the expression of one gene in *N. crassa* in response to changing environmental conditions [12]. In animals, a preliminary study has indicated that H1 may play a role in the activation of apoptotic pathways [201], and two more recent studies have also shown transcriptional changes arising from H1 depletion, including broad changes to *hox* gene expression in mice [202], and to CHD8 modulated repression of Wnt-β-catenin transcription in HeLa cells [203]. It is not clear if this is because H1 exhibits only a limited impact on transcription, despite its almost ubiquitous presence in chromatin, or if the knockdown studies failed to achieve sufficient depletion of H1. To investigate H1's role on transcriptional regulation in *N. crassa*, global transcriptional changes induced by the loss of histone H1 was investigated by mRNA-sequencing. This was done in both the WT (S1) and the H1 deletion (*Δhho*) strains.

As a result of these experiments we have identified a subset of genes whose transcript levels are affected by the loss of H1. All genes in this subset have altered

mRNA transcript levels, resulting in either an up-, or down-regulation in the deletion mutant (*Δhho*) by a minimum log 2 value as seen in Figure 1. An example of this expression change for an individual gene locus can be seen in Figure 2. Additional analysis will be required to determine if this subset of genes appear to have any general metabolic character, as the effected genes are spread across a wide variety of metabolic functions based on preliminary identification by current annotations as shown in Table 2.

Figure 1: Histogram of Log 2 expression changes in mRNA-seq data set. The histogram shows all the log 2 transcript levels found in the data set for the WT (S1) strain in the mRNA-seq study as plotted in R (Alexander Matte Santos, unpublished) [199]. The X axis shows log 2 transcript levels, and the Y axis shows frequency of genes that have a specific log 2 transcript level value. The expression values for all genes are shown in grey, those genes up-regulated in *Δhho* are shown in red, and those down-regulated in *Δhho* are shown in blue.

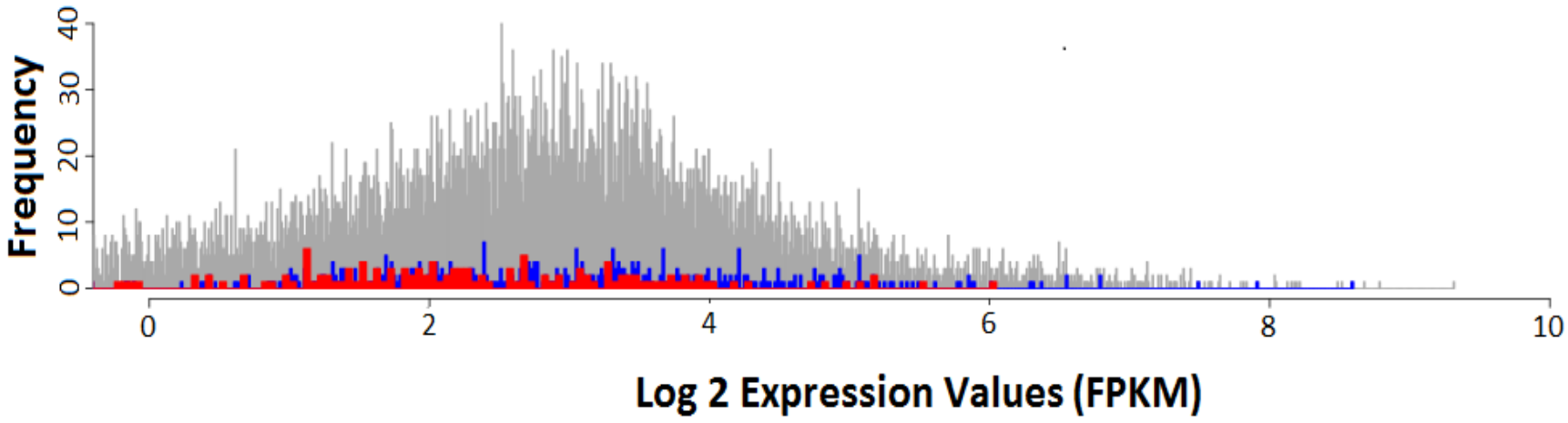


Figure 2: Example of mRNA-seq expression differences on linkage group V. This figure shows a visualization of mRNA transcript expression differences between the WT (S-1, blue) and H1 deletion (*Δhho*, red) strains at an example locus on Linkage Group V as visualized on IGV viewer [204]. The X axis shows a plot of Linkage group V with genes annotated at the bottom, while the Y axis represents relative read number in the mRNA data set. Brown boxes denote gene loci with no significant difference in transcript levels. The red box indicates a gene found to be down regulated in the deletion strain (*Δhho*). The blue box indicates a gene up-regulated in the deletion strain (*Δhho*).

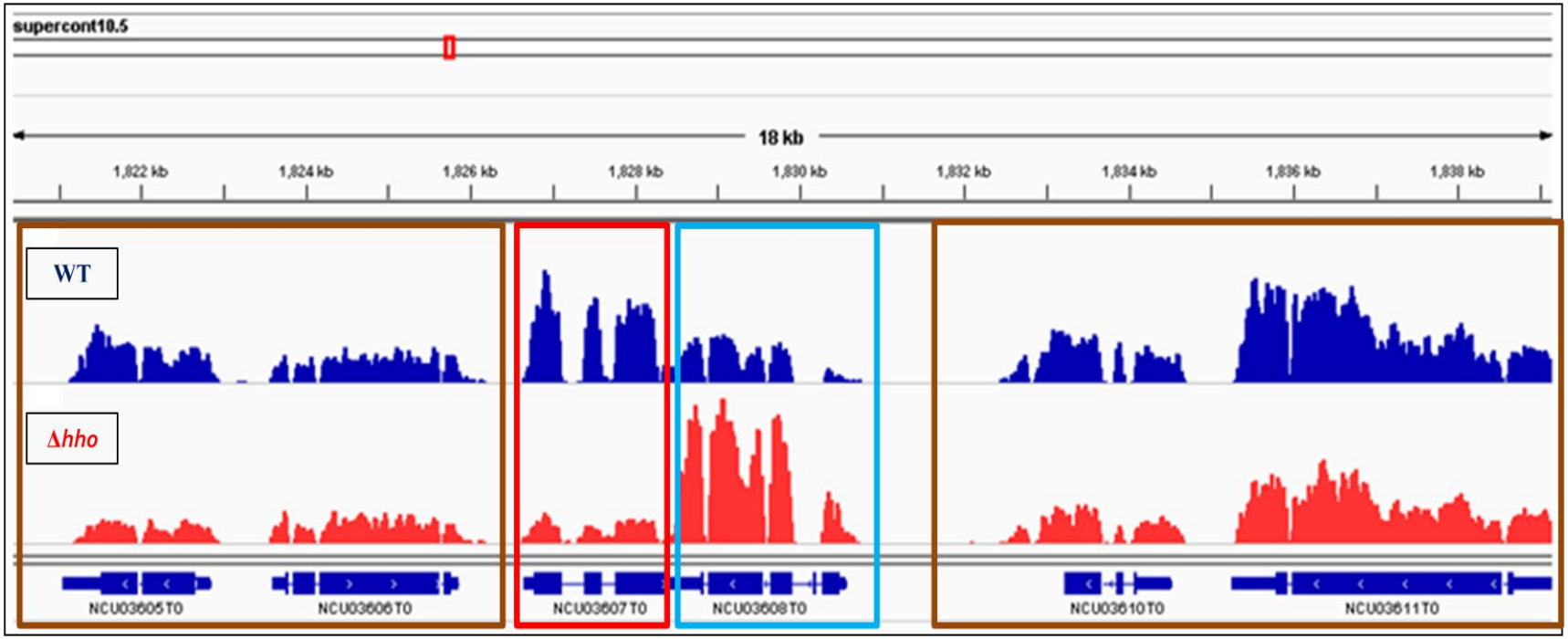


Table 2: Δ*hho* transcriptional differences. Metabolic pathways found to contain genes differentially expressed in the H1 deletion mutant (*Δhho*) of *N. crassa*. This table shows the total number of genes found to be mis-regulated in expression level in the mRNA-seq data set by metabolic category.

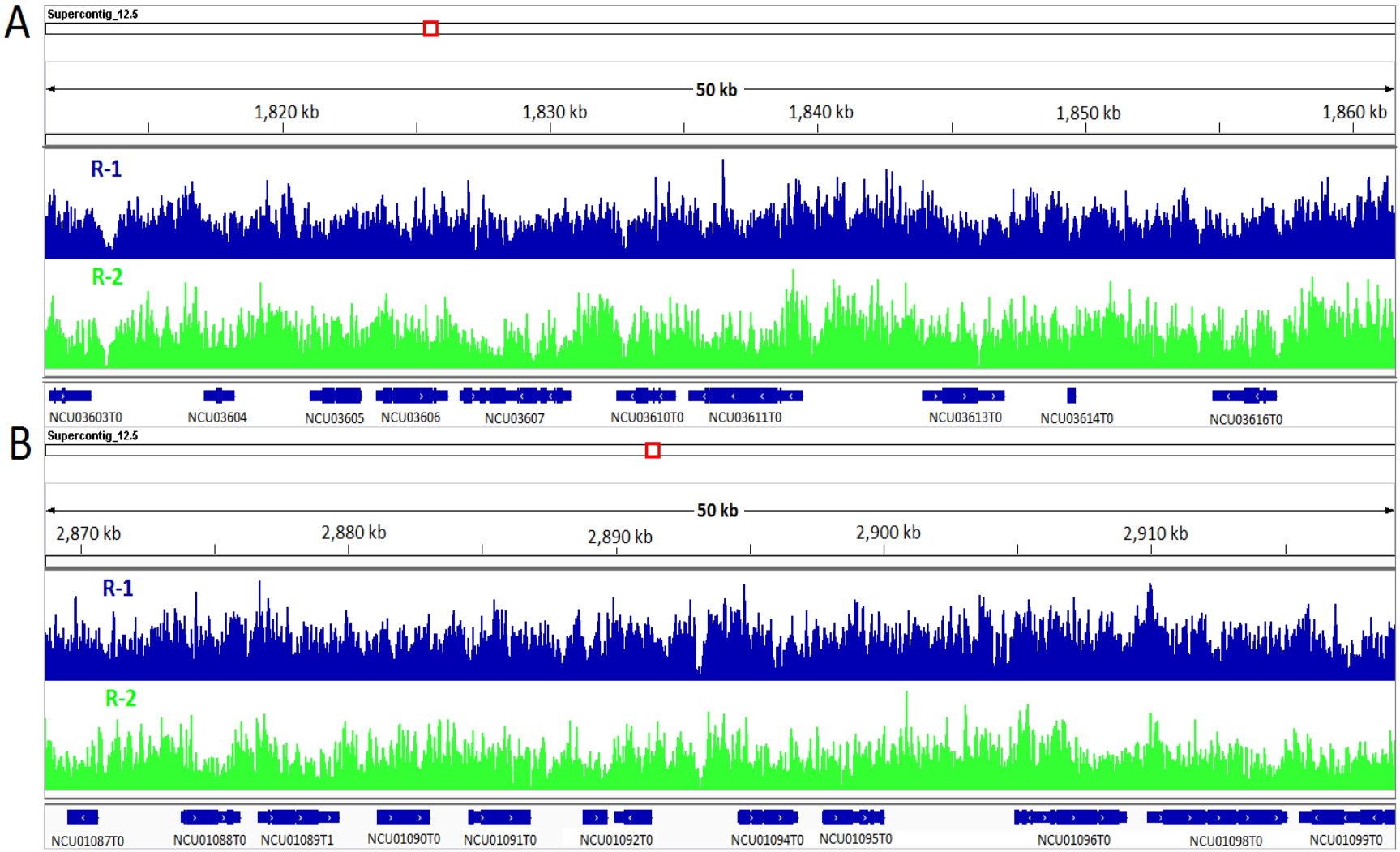
Metabolic pathway of affected genes	**Increased expression in *Δhho***	**Decreased expression in *Δhho***
Amino Acid Biosynthesis	**14**	**3**
Cell Wall / Membrane Maintenance	**0**	**4**
Cell Wall / Membrane Transport	**0**	**6**
Energy Cycling	**0**	**7**
Histone Biosynthesis	**1**	**0**
Light Response	**0**	**1**
Lipid Metabolism	**3**	**2**
Nucleotide Metabolism	**3**	**2**
Mitochondrial DNA maintenance	**1**	**0**
Protein metabolism	**2**	**3**
Ribosome Biogenesis	**2**	**0**
Secondary Metabolite Synthesis	**0**	**3**
Sexual Reproduction	**0**	**1**
Sugar Metabolism	**0**	**10**
Possible Virulence Factor	**1**	**4**
Unknown gene function	**18**	**57**
Total	**44**	**104**

ChIP-Seq

H1 occupancy of chromatin in *N. crassa* appears to be global, with no statistically significant differences in occupancy across both euchromatin and heterochromatin regions. As described above, chromatin and the core histones have been shown to regulate gene activity. This is due to the character of the chromatin region in which a gene locus is found (heterochromatic vs. euchromatic) and to the PTMs found on the core histone proteins at a given locus [64, 65]. Extensive studies have been done on the core histones, and early research on histone H1 indicates that it also plays a role in the regulation of both chromatin compaction and higher-order chromatin structures [66, 141, 145]. Since the core histones exhibit a highly regulated pattern of chromatin occupancy, it is reasonable to expect the same of H1. Since H1 binds genomic DNA where it enters and exits the nucleosome, it may play a role in regulating linker DNA length and nucleosome stability [1, 3-5, 8, 61, 63]. To investigate H1's distribution and any possible occupancy pattern on the genome of *N. crassa* a series of ChIP-seq studies were conducted. These experiments were done in the 3X FLAG-tagged H1 mutant strain LX-44-5 (Lewis, unpublished)

As a result of these experiments it was discovered that H1 has no specific pattern of occupancy on the *N. crassa* genome, and instead appears to be distributed globally with no statistical difference between euchromatic and heterochromatic regions. This is also true for individual gene loci, where there appears to be no difference in H1 occupancy across the UTRs or CDS as seen in Figure 3. Confirmation of these data was done by qPCR of ChIP-seq samples targeting genes found to be mis-regulated in the mRNA data sets (data not shown).

Figure 3: Examples of H1-ChIP occupancy of linkage group V. This figure shows a visualization of H1 ChIP-seq reads in the 3xFLAG tagged H1 (LX-44-5) strain at two different loci on Linkage Groups V as visualized in IGV viewer [204]. The X axis shows a plot of Linkage group V with genes annotated at the bottom, while the Y axis represents relative read number in the H1 ChIP-seq data set. The top section (A) shows two independent biological replicates (blue and green) for the area around NCU03610. The bottom section (B) shows two independent biological replicates (blue and green) for the area around NCU01092. No statistically significant differences in H1 occupancy were found.


A
Supercontig_12.5
50 kb
1,820 kb
1,830 kb
1,840 kb
1,850 kb
1,860 kb
R-1
R-2
NCU03603T0
NCU03604
NCU03605
NCU03606
NCU03607
NCU03610T0
NCU03611T0
NCU03613T0
NCU03614T0
NCU03616T0
B
Supercontig_12.5
50 kb
2,870 kb
2,880 kb
2,890 kb
2,900 kb
2,910 kb
R-1
R-2
NCU01087T0
NCU01088T0
NCU01089T1
NCU01090T0
NCU01091T0
NCU01092T0
NCU01094T0
NCU01095T0
NCU01096T0
NCU01098T0
NCU01099T0

MNase-Seq

Loss of histone H1 results in some nucleosomal instability in the subset of genes found to be mis-regulated in the mRNA-seq study and that quartile of genes with the lowest expression levels in the WT (S1) strain**.** Extensive studies have been done on the core histones, and early research on histone H1 indicates that it plays a role in the regulation of both chromatin compaction and higher-order chromatin structures [66, 141, 145]. Since H1 binds genomic DNA where it enters and exits the nucleosome, it is believed that it may play a role in regulating linker DNA length and in the stabilization of nucleosomes on the genome [1, 3-5, 8, 61, 63]. Micrococal Nuclease digestion degrades unbound linker DNA, however, DNA bound by the nucleosome is protected and does not initially undergo digestion by this nuclease. By sequencing the DNA that remains following MNase digestion of cross-linked DNA, nucleosome occupancy across the genome can be mapped [11, 191, 192]. To investigate any changes that may occur in nucleosome occupancy or stability in the absence of H1 in *N. crassa* a series of MNase-seq studies were conducted. These studies were done in both the WT (S1) and H1 deletion (*Δhho*) strains.

As a result of these experiments it was discovered that there appear to be some changes in nucleosomes stability around the Nucleseosome Free Region (NFR) in the subset of genes found to be mis-regulated in the mRNA-seq study (see above). An individual example of this effect at a single gene locus can be seen in Figure 4. It was also noticed that MNase digestion of chromatin down to single nucleosome fragments may occur somewhat faster in the H1 deletion mutant (*Δhho*) in comparison to the WT (S1) strain when visualized on an 2% agarose gel as shown in Figure 5. Meta-genomic

analysis of the MNase-seq data for the mis-regulated mRNA-seq gene set shows a decrease in average reads in the +1 Nucleosome in the H1 mutant (*Δhho*) in contrast with the WT (S1) strain as shown in Figure 6. This is most readily apparent in the gene quartile with the lowest expression in the WT (S1) strain as shown in Figure 7. A slight change in read number in the +1 nucleosome region was also found in the other expression quartiles of this set, however, it was much less pronounced.

Figure 4: Example of MNase-seq read data at gene locus NCU01092. The top four lines in the diagram are a visualization of mRNA transcript levles between the WT (S-1, blue) and H1 deletion (*Δhho*, red) strains at example locus NCU01092 on Linkage Group V as visualized on IGV viewer [204]. The bottom four lines in the diagram are a visualization of MNase-seq reads for both the WT (S-1, blue) and H1 deletion (*Δhho*, red) strains at example locus NCU01092 on Linkage Group V as visualized on IGV viewer [204]. The X axis shows a plot of Linkage group V with genes annotated at the bottom, while the Y axis represents relative read number in the mRNA-seq and MNase-seq data sets respectively. The Red boxes denote changes in nucleosome occupancy between the WT (S1) and H1 deletion (*Δhho*) strains in the Nucleosome Free Region (NFR) of this gene in the MNase-seq data set.

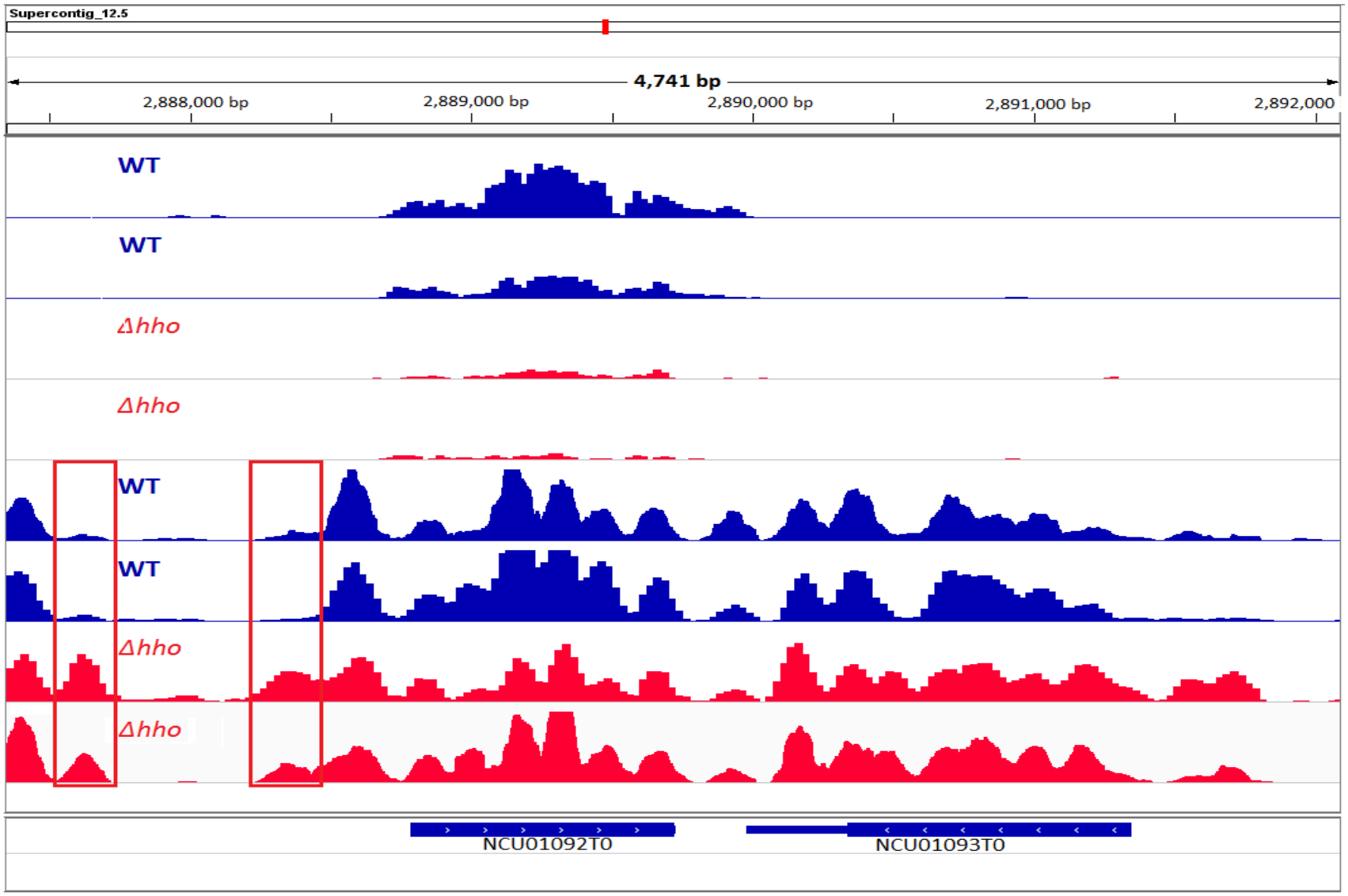

Supercontig_12.5
4,741 bp
2,888,000 bp
2,889,000 bp
2,890,000 bp
2,891,000 bp
2,892,000
WT
WT
Δhho
Δhho
WT
WT
Δhho
Δhho
NCU01092T0
NCU01093T0

Figure 5: Differences in WT and *Δhho* MNase digestion patterns. This figure shows the results of the MNase digestion of cross-linked DNA of the WT (S1) and H1 deletion mutant (*Δhho*) strains of *N. crassa* for various digestion time intervals as visualized on a 2% agarose gel. Gel lanes were loaded with 250ng of DNA per lane and visualized with EtBr under UV light. DNA ladder used was Invitrogen 1Kb plus (Thermo Fisher Scientific Inc., MA Cat #10787-018) and enzyme digestion times for the lanes are as follows: 1) Ladder 2) no enzyme control 3) 1 minute 4) 5 minute 5) 10 minute 6) 20 minute 7) 40 minute. The red boxes show the samples with 10 and 20 minutes of MNase digestion where the *Δhho* samples show a higher intensity of signal in the lower bands. These differences may indicate increased accessibility of MNase to the DNA in the *Δhho* samples due to deficits in nucleosome stability, resulting in faster digestion of both euchromatic and heterocromatic regions.

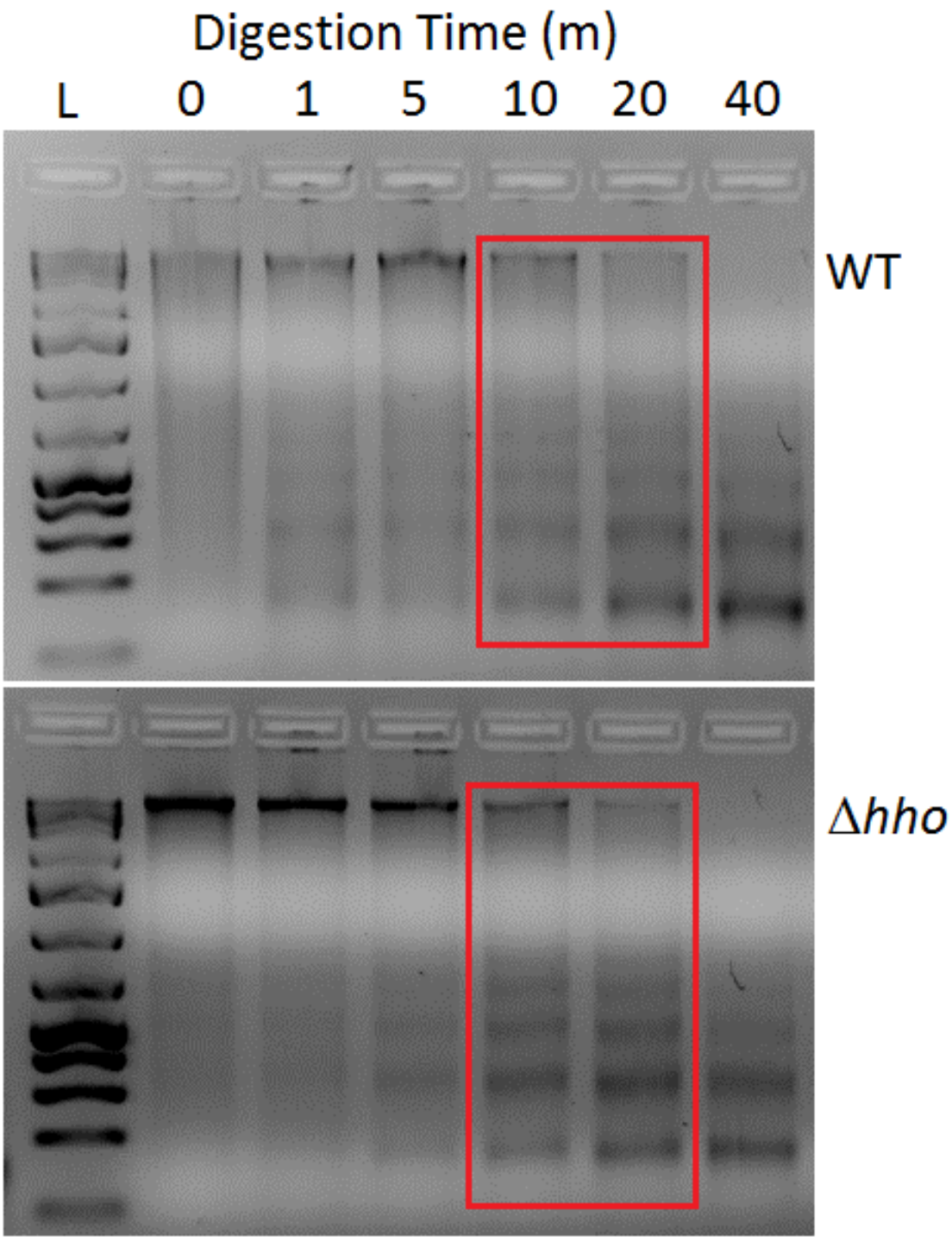
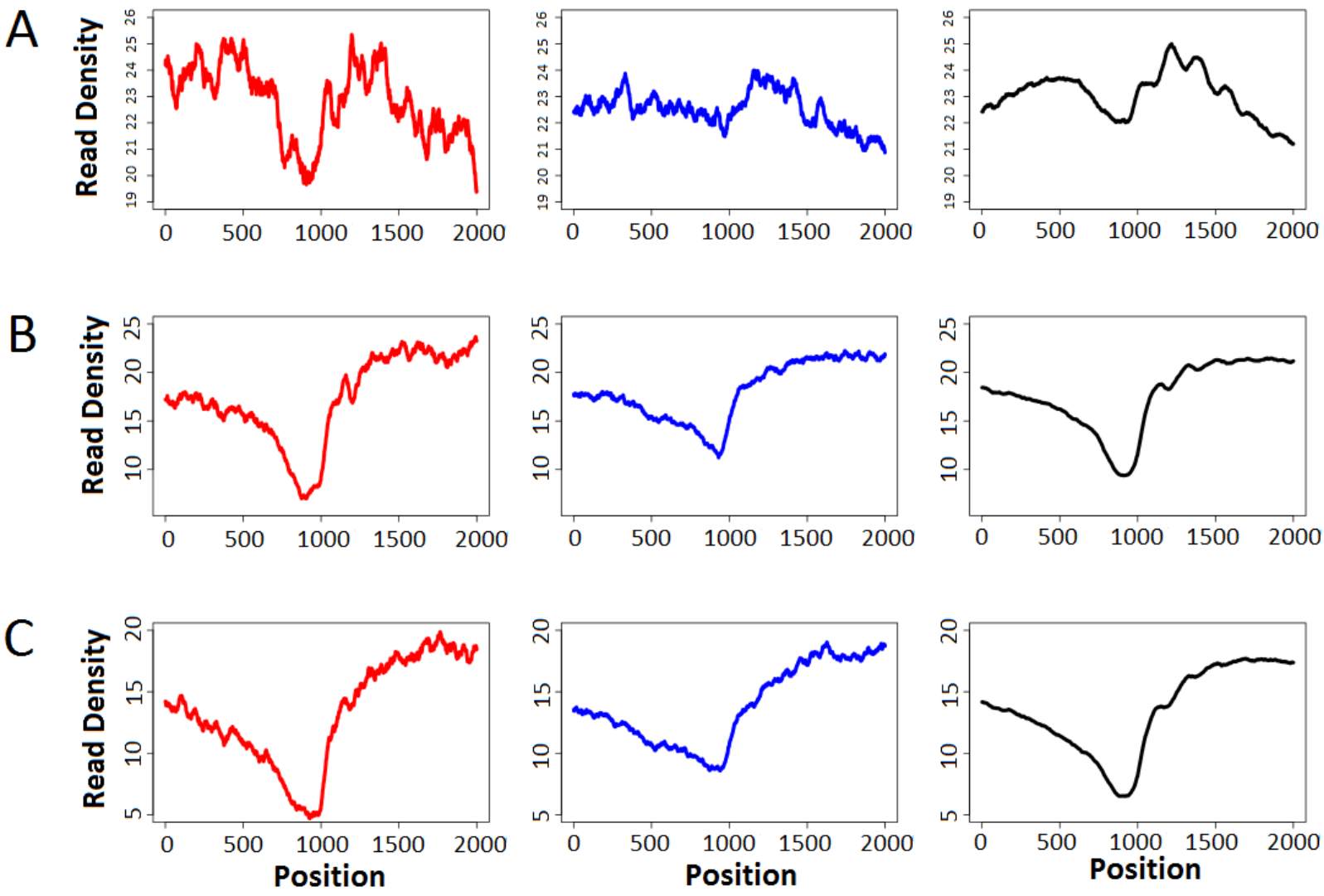
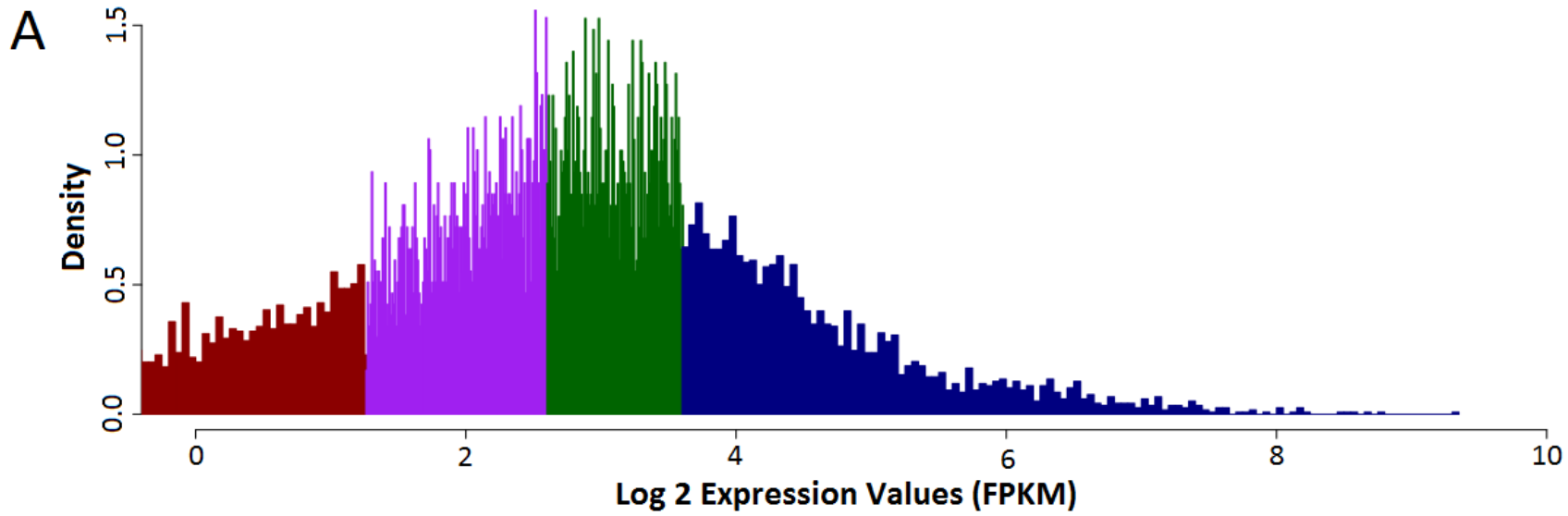

Digestion Time (m)
L
0
1
5
10
20
40
WT
Δhho

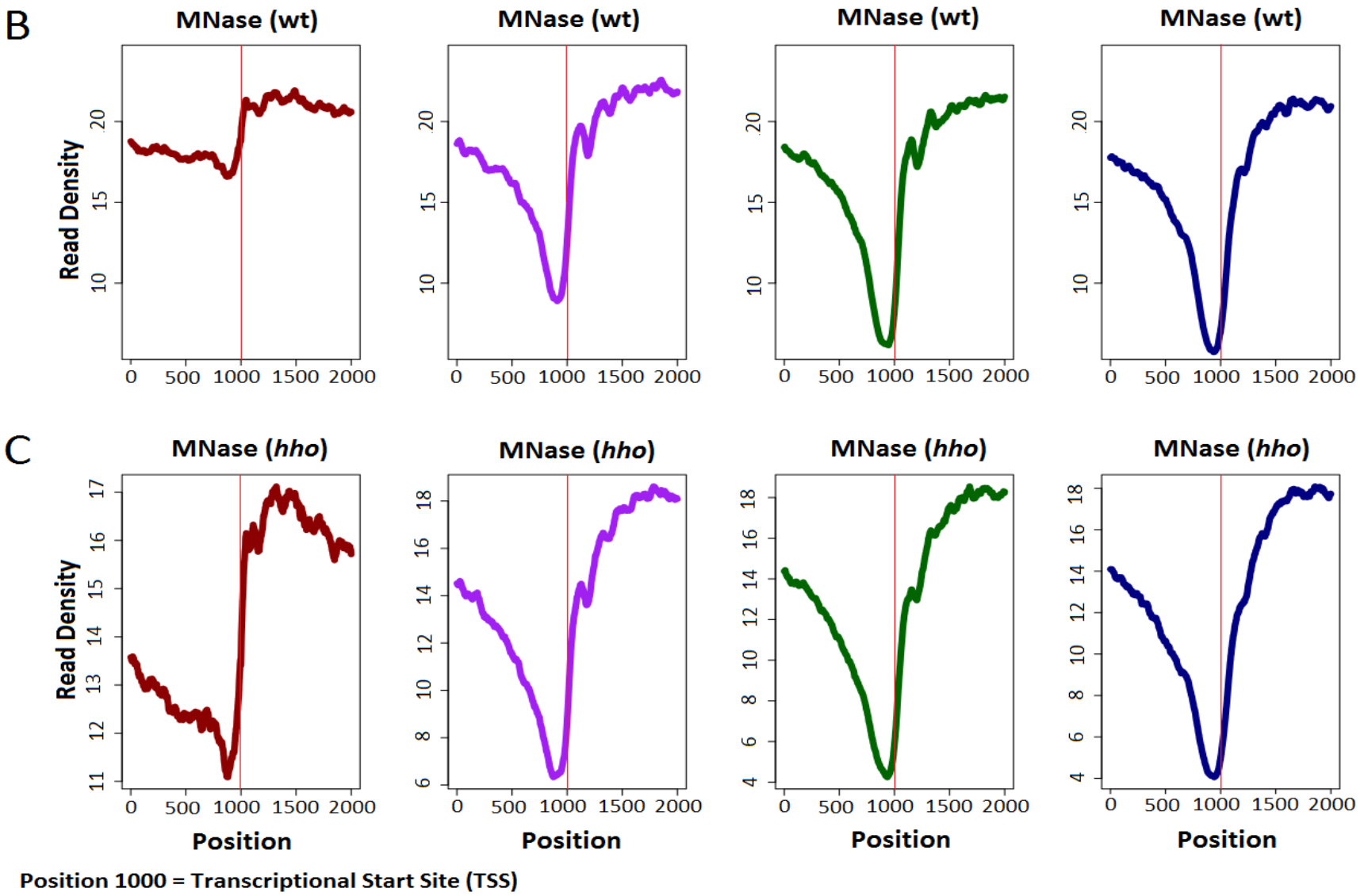
Figure 6: MNase-seq read levels in the mis-regulated mRNA gene set. This figure shows changes in average MNase-seq read intensity for the gene set found to be mis-regulated in the mRNA study as as plotted in R (Alexander Matte Santos, unpublished) [199]. In these figures the Y axis shows relative read number and the X axis shows an averaged gene locus with the TSS at 1000. Graphs with red lines represent an average of the subset of genes up-regulated in *Δhho*, graphs with blue line represent an average of those genes down-regulated in *Δhho*, and graphs with black lines shows an average off all genes. The top row of graphs show average read intensity for the H1 ChIP-seq study (A). The middle row of graphs show average read intensity for the MNase-seq data set in the WT (S1) strain (B). The bottom row of graphs show average read intensity for the MNase-seq data set in the H1 deletion (*Δhho*) strain (C).



Position 1000 = Transcriptional Start Site (TSS)

Figure 7: Example of changes in MNase-seq reads levels in the NFR. The histogram at the top of the figure shows changes in log 2 transcript levels for the WT (S1) strain in the mRNA-seq study as plotted in R (Alexander Matte Santos, Unpublished) [199]. The X axis shows log 2 transcript levels, and the Y axis shows frequency of genes with a particular log 2 transcript level as in Figure 1 above. The WT (S1) mRNA-seq gene set has been split into quartiles, and color coded by increasing log 2 expression values (A). The first row of graphs shows the MNase-seq read level across an averaged gene locus for each quartile in the WT (S1) strain (B). The second row of graphs shows the MNase-seq read level across an averaged gene locus for each quartile in the H1 deletion (*Δhho*) strain (C). In both (B) and (C) the Y axis shows relative read number and the X axis shows an averaged gene locus for the quartile with the TSS at 1000 (denoted by the thin vertical red line) as plotted in R (Alexander Matte Santos, unpublished) [199].




B
MNase (wt)
MNase (wt)
MNase (wt)
MNase (wt)
Read Density
C
MNase (*hho*)
MNase (*hho*)
MNase (*hho*)
MNase (*hho*)
Read Density
Position
Position
Position
Position
Position 1000 = Transcriptional Start Site (TSS)

CHAPTER 5

DISCUSSION

To identify the role H1 plays in the regulation of transcript population and chromatin structure of *N. crassa*, studies were conducted to identify the normal occupancy pattern of H1 on the genome and to identify if its loss had any effect on nucleosome stability or transcriptional regulation. Our investigations revealed no differences in H1 occupancy, however, in the mRNA-seq study it was discovered there were changes in the transcript levels of a small set of genes in the H1 deletion (*Δhho*) strain. Of the genes mis-regulated in the H1 deletion (*Δhho*) strain a subset had significant changes in their expression levels, with a change in transcript level of log 2 or more. In addition to altered gene transcription levels found in the mRNA-seq study, the MNase-seq data set revealed that many of these mis-regulated genes show distinct changes in nucleosome occupancy, often in and around the Nucleosome Free Region (NFR). This is significant because histone H1 has been found to play a role in chromatin structural stability in previous studies, and serves to establish a parallel between *N. crassa* and higher eukaryotes [65, 143]. Though no specific H1 localization patterns were found in the ChIP-seq studies, the existence of a specific mis-regulated gene set in the H1 deletion (*Δhho*) strain would indicate that H1 is playing a role in their regulation.

Though the initial mRNA-seq studies have successfully shown transcriptional changes in a defined gene set in the H1 deletion (*Δhho*) strain, it is currently unknown if this mis-regulation is directly caused by the binding of H1 itself, or if it arises due to a

cascade effect caused by H1's interaction (or lack thereof) with transcriptional factors or chromatin remodeling proteins higher in the regulatory chain. In addition, since this study looked at only the WT (S1) and H1 deletion (*Δhho*) strains, it might be productive to examine the effects of either a partial depletion or over expression of H1 on this gene set in future studies. A partial depletion or over expression study would allow investigators to directly control the relative levels of H1 expression, which would help to define the minimum and maximum levels of H1 protein required for normal function in *N. crassa*. This could be done through the use of RNA interference pathways or inducible / alternate promoter constructs [205, 206].

There is also that possibility that some of the altered transcript levels in the H1 deletion (*Δhho*) strain result from cryptic transcription caused by the destabilization of chromatin structure as evidenced in the differences found in the MNase-seq data sets. Cryptic transcription is the expression of non-coding RNA (ncRNA) or anti-sense RNA (asRNA) from non-standard promoters within a gene locus [207, 208]. It is possible that H1's association with chromatin plays a role in suppressing cryptic promoters by stabilizing nucleosome positioning and higher order chromatin structure [209, 210]. Due to the fact that the RNA-seq experiments conducted in this study focused on mRNA transcription by isolating polyadenylated transcripts, it may be advisable to repeat this sequencing study on the total RNA sample. Any sequencing libraries constructed from the total RNA sample should reveal the presence of any ncRNA or asRNA resulting from active cryptic promoters. It may also be possible to utilize strand-specific RNA-seq or qPCR to confirm the results of any changes found in such a study [211, 212].

Previous studies in *N. crassa* also found that the loss of H1 induced transcriptional changes under alternate growth conditions [12]. Another future study should be done to explore variations in transcriptional expression in greater detail in both the WT (S1) and H1 deletion (*Δhho*) strains under such alternate growth conditions. The effects of nutritional stress on the WT (S1) or H1 deletion (*Δhho*) strain can easily be examined by varying nutrient availability in the standard growth media, or through the addition of chemical agents to induce various forms of environmental stress.

Since this study provides a reliable baseline expression level of the *N. crassa* genome for both the WT (S1) and the H1 deletion (*Δhho*) strains under normal growth conditions, it may also prove worthwhile to investigate any possible effects of H1's loss on various differentiated cell types or in cells at different points in the cell cycle. Though the isolation of small populations of specific cell types (such as a parathecia) or cells in a certain phase of the cell cycle is fairly difficult, it may prove more practical in the future as both cellular isolation and sequencing technologies advance.

Another avenue by which the control of this mis-regulated gene set might be effected is through the post-translational modification of H1. As discussed in the introduction, preliminary studies have shown that H1 may be regulated in a similar fashion to the core histones. H1 regulatory mechanisms, such as PTMs, competitive inhibition, and various protein-protein interactions, have been found in a variety of model organisms including humans, mice, and Drosophila [150, 201, 213]. The specific regulation of histone H1 is demonstrated most clearly in *Mus musculus*, where multiple H1 genes are expressed differentially, in both a cell cycle and tissue-dependent manner [150, 151]. Though *N. crassa* has only a single H1 gene, it seems reasonable to expect

that it too will be tightly regulated through the same type of control pathways seen in other eukaryotes. Though attempts to purify H1 were unsuccessful during the term of this study, future investigators could work to identify any PTMs present on H1 in *N. crassa* through the use of mass spectrometry analysis of purified H1samples utilizing the proven LX-44-5 strain (Lewis, unpublished) [157, 214]. This would allow for the comprehensive identification of any H1 PTMs present in the H1 protein population of *N. crassa*.

A set of preliminary experiments done in this lab have also shown this same LX-44-5 strain could also be used to identifying H1 protein-protein interactions present in *N. crassa* through the use of co-immunoprecipitation assays (Lewis, unpublished data). Though these initial results showed a preponderance of ribosomal proteins, it is possible that the protein isolation procedure may not have been stringent enough to remove the bulk of non-specific chromatin binding proteins from the sample analyzed. To rectify this problem, future investigators could perform additional co-immunoprecipitation assays utilizing high salt conditions to avoid non specific protein-protein interactions [215]. Alternate or complimentary studies could also be done utilizing yeast-2-hybrid or bi-molecular fluorescence assays [216, 217].

Though both H1's occupancy across the genome and its effect on nucleosome positioning has been examined over the course of this study, the results reported here represent only an initial foray into understanding the functionality of H1 in *N. crassa*. The studies presented here have succeeded in laying a strong foundation for future experimentation and have established *N. crassa* as a viable model organism for the investigation of H1 with clear parallels to higher eukaryotic organisms.

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