DEVELOPMENT OF MOLECULAR TOOLS TO MONITOR DNA REPLICATION IN HETEROCHROMATIN-DEFICIENT MUTANTS

by

ASHLEY L. HAGEN

(Under the Direction of Zachary Lewis)

ABSTRACT

The *Neurospora crassa* H3K9 histone methyltransferase DIM-5 is required for heterochromatin formation, DNA methylation, and normal genome maintenance. Critical components of genome maintenance include DNA replication and repair, and cytological defects in these processes result in genome instability, a known hallmark of cancer. Recent evidence suggests that DIM-5 might have a role in the regulation of DNA replication. The purpose of this study is to develop new molecular tools to monitor DNA replication in heterochromatin-deficient mutants, and the long-term goal of this research is to better understand how eukaryotic organisms preserve the integrity of complex genomes.

INDEX WORDS: DIM-5^{KMT1} (Defective in methylation-5), histone methyltransferase, genome maintenance, genome instability, DNA replication, nascent strand, fluorescent reporter construct, photoconvertibility, *Neurospora crassa*

DEVELOPMENT OF MOLECULAR TOOLS TO MONITOR DNA REPLICATION IN HETEROCHROMATIN-DEFICIENT MUTANTS

by

ASHLEY L. HAGEN

BS, University of Georgia, 2012

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

2016

DEVELOPMENT OF MOLECULAR TOOLS TO MONITOR DNA REPLICATION IN HETEROCHROMATIN-DEFICIENT MUTANTS

by

ASHLEY L. HAGEN

Major Professor:	Zachary Lewis
Committee:	Timothy Hoover
	Vincent Starai

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
December 2016

DEDICATION

This work is dedicated to my family. To my grandparents for being the first in my life to exemplify the love of science and passion for life long learning. For always being prepared to engage with me in scientific discourse or challenge me with puzzles, riddles and informed debates. For being my pioneers, teachers, adventurers, advisors and encouragers. To my parents for always being my biggest supporters and acting as my most constant examples of determination, hard work and independence. For truly loving me unconditionally and seeing potential in me first. For encouraging me to be independent and equally strong in heart and mind. For showing me how to dig deep when I have needed it most, teaching me the value of a job well done and reminding me that the smartest way is one step at a time. To my brother for being my protector, encourager, friend, and fellow UGA Alumni! For making me smile or laugh countless times on this journey when I desperately needed it. For offering constant support and love, and for your regular display of strength and dependability in my life. I am so proud of you. To my husband for loving and believing in me daily. For sharing in my passion for science and for challenging me to be growing regularly. For supporting my educational and occupational choices and encouraging me to do what I love. For understanding my drive and determination in this pursuit even when circumstances have made it challenging for us both. I love you all very much. This would never be possible without each one of you.

ACKNOWLEDGEMENTS

I would like to thank my major professor and advisor Dr. Zachary Lewis for his guidance and support as my teacher, advisor, and advocate. I would specifically like to thank Dr. Lewis for teaching me how to be a better scientist, improve my critical thinking skills and problem solve creatively. I would also like to thank Dr. Lewis for supporting and advocating for me to succeed in all of my educational and occupational pursuits. I would like to thank my committee members, Dr. Timothy Hoover and Dr. Vincent Starai, for supporting me and advising me throughout my undergraduate and graduate work at the University of Georgia. I would like to thank them for helping to shape my scientific development and for giving constructive direction over the years. I would also like to thank the Department of Microbiology for standing on a principle of student advocacy and excellence and acknowledge the Graduate School for financial support. Finally, I would like to thank the Department of Microbiology faculty members and my classmates for their academic influences and friendship. What I will remember most fondly about my time at UGA is the wonderful relationships that I formed here.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

DNA replication and repair are critical components of genome maintenance, and cellular defects in these processes can result in genome instability, a known hallmark of cancer [1-4]. In eukaryotes, the relevant substrate for most DNA-based processes is chromatin, a highly organized complex of DNA and proteins [4-6]. Local chromatin structure can be modified by attachment of small chemical modifications to DNA or to chromatin proteins [3-5,7-9]. These chromatin modifications can impact virtually all DNA-templated processes in the nucleus, [3-5,7-9], providing a level of control that is independent of DNA sequence. This regulation is referred to as epigenetic, to reflect the fact that chromatin modifications are relatively stable, yet still flexible to the changing needs of the cell [1,3,4]. Disruption of epigenetic regulators is widespread in cancer cells and pervasive in malignancy, demonstrating the importance of chromatin structure and function [3,4]. Therefore, epigenetic control mechanisms are critical to genome maintenance, and understanding how these mechanisms are involved in the promotion of faithful DNA replication and repair in eukaryotic cells is of great clinical significance.

Eukaryotic DNA is packaged in two cytologically and functionally distinct forms of chromatin, euchromatin and heterochromatin [1,10,11]. Euchromatin is loosely packaged DNA that is typically transcriptionally active while heterochromatin is more tightly packaged and associated with regions of the genome that are transcriptionally

inactive or silent [1,10,11]. Heterochromatin is concentrated in pericentromeric and telomeric regions of most eukaryotic chromosomes [1], and despite its repeat-rich and gene poor nature, heterochromatic modification pathways play critical functions in numerous essential cytological operations, such as centromere formation and transposon silencing [1,10]. Effective DNA replication must occur in all chromatin environments to ensure faithful transmission of genetic information from one generation to the next.

The conserved histone H3 lysine K9 (H3K9) methyltransferase, DIM-5^{KMT1} (Defective in methylation-5), is necessary for heterochromatin formation and genome maintenance in the filamentous ascomycete fungus *Neurospora crassa* [10-12]. Furthermore, the promotion of genome maintenance is a conserved role of DIM-5 homologs in *Schizosaccharomyces pombe, Drosophila melanogaster* and mice [10,11,13]. Available data suggest that deletion of *dim-5* may lead to defects in DNA replication, DNA repair or chromosome segregation [10,11,13].

As will be supported in the following literature review, I hypothesize that DIM-5 contributes to genome maintenance by promoting faithful DNA replication in *N. crassa*. **The objective of my thesis work was to develop new tools to monitor DNA replication in both wild-type and *Δdim-5* strains of *N. crassa* cells in order to examine the validity of my hypothesis.**

I will begin by summarizing a few of the important characteristics of the organism *N. crassa* that make it an ideal model system for the study of epigenetics. Next I will provide a short review of eukaryotic DNA packaging and chromatin structure, followed by a more in-depth explanation of the known functions of DIM-5, as well as the data supporting a possible role for this enzyme in promoting DNA replication. I will then

provide a review of eukaryotic DNA replication with a focus on origins of replication, origin priming and firing at the initiation of replication. Along the way, I will examine functional correlations of DIM-5 homologues in other organisms. I will discuss similarities in the regulation of replication in *N. crassa, Saccharomyces cerevisiae* and *S. pombe.* Finally, I will speculate on mechanisms for DNA replication control in filamentous ascomycetes.

The work presented here details two newly developed DNA replication-monitoring techniques in both wild-type and *Δdim-5 N. crassa* cells. The first is a nascent strand DNA sequencing assay designed to identify origins of replication by harvesting asynchronously dividing cells during logarithmic growth and isolating short nascent DNA strands (SNS) from these cell cultures for Illumina sequencing. The second approach is designed to monitor the time required for individual nuclei to complete S-phase by developing a photoconvertable fluorescent reporter construct. In concert, the goal is to gain an understanding of how and where DNA replication begins in this eukaryotic model organism, as well as how long DNA replication takes to progress. In so doing, I am attempting to characterize the role of DIM-5 in the promotion of faithful DNA replication and the maintenance of genome stability.

CHAPTER 2

LITERATURE REVIEW

Neurospora crassa

The filamentous ascomycete fungus, *N. crassa*, maintains a central role as a model organism for studies of chromatin structure and epigenetics. Its moderate genome size and genetic tractability make *Neurospora* a very desirable choice in the laboratory. At about 45 megabases in size and 20% repeated DNA content, the *N. crassa* genome is significantly larger and more complex than *S. cerevisiae* and *S. pombe* (both about 12Mb with 1% repeated DNA) [14]. Higher eukaryotes sport much larger genome sizes with considerable repeated content, making them more difficult to manipulate in a laboratory setting. The moderate genome size and complexity of *N. crassa* is small enough to be amenable to cheap and easy sequencing-based approaches [14]. Furthermore, with an available whole-genome knockout library and fast growth rate (about 3-4 mm per hour at 25°C) under simple nutritional requirements, *N. crassa* is relatively economical to culture and manipulate [15,16].

Additionally, the organization of the *N. crassa* genome is more similar to higher eukaryotes than that of the model yeasts. For example, only 3% of the *S. pombe* genome is packaged into heterochromatin [17], whereas the genomes of higher eukaryotes such as *D. melanogaster* and humans have heterochromatin compositions >30% [18,19]. The *N. crassa* genome is ~18% heterochromatin and includes centromeres, subtelomeres and dispersed domains along chromosome arms, making it a tractable model system for

studying heterochromatin structure [1,14]. Additionally, *Neurospora* possesses many conserved molecular features of heterochromatin that are present in higher eukaryotes but absent in yeast. These include DNA methylation, H3K9 methylation, and Heterochromatin Protein 1 (HP1) [7,14]. Therefore, *N. crassa* offers a manageable genome for epigenetic study that contains many important similarities to the genomes of higher order eukaryotes.

DNA Packaging

A foundational knowledge of chromatin structure is necessary before epigenetic regulation can be understood. The typical eukaryotic nucleus ranges between 10-20 microns in diameter [6], yet it must house an entire genome within its borders. The DNA must therefore be condensed to fit inside the nucleus. The process of DNA condensation is referred to as DNA packaging, and the basic, repeating unit of packaged DNA is the nucleosome. A nucleosome consists of approximately 146bp of DNA wrapped approximately 1.7 times around a core histone octamer and effectively shortens the DNA fiber length by about seven-fold [6]. Nucleosomes are liked together via ~20 bp of "linker DNA" [6]. When viewed under electron microscopy, nucleosomes joined by linker DNA give the appearance of beads on a string. Chromatin is then coiled into higher-order structures, such as 30 nm fibers, in order to further condense the genome. Amazingly, at its completion, DNA packaging is an effective method of condensing the genomes of eukaryotic organisms to sizes that do, in fact, fit inside their corresponding nuclei.

A closer look at the core of the nucleosome illuminates how critical histone proteins are to the structure and function of DNA packaging. Histones are positively

charged proteins that attract and tightly bind negatively charged DNA [6]. There are four conserved core histone proteins, H2A, H2B, H3, and H4. Each nucleosome consists of a histone octamer, comprised of two copies of each of the four core histone proteins [6]. Importantly, the N-terminal tails of histones are not a part of the histone core, meaning that steric hindrance is eliminated in these regions and the amino acid residues of the histone tail are accessible to enzymatic modification. Post-translational histone modifications, along with direct DNA modification, compose the core mechanisms of epigenetic control.

Chromatin

The eukaryotic genome is packaged in two cytologically and functionally distinct chromatin environments [1,10,11]. While euchromatin is gene-rich and exists in an open conformation allowing easy access of transcriptional machinery, heterochromatin remains conformationally compact and transcriptionally silent. Additionally, constitutive heterochromatin typically replicates late in S phase and contains hypermethylated domains in organisms that exhibit DNA methylation [12].

Furthermore, heterochromatin tends to be gene-poor, but A:T and repeat-rich [1,10-12]. Because of this supposed lack of genetic content, heterochromatin was historically labeled “junk DNA.” However, recent findings have demonstrated that transcriptional inactivity of heterochromatin domains does not correlate to a lack of function [1,10,11]. In fact, heterochromatin is believed to play a pivotal role in genome maintenance, and proper heterochromatin formation and regulation is required for a variety of normal cell cycle operations that do not rely on gene coding capacity. Regulation of proper centromere formation and function, normal chromosome

organization, transposon silencing, and telomere protection are all documented functions of heterochromatin [1,10,11].

Post-translational Histone Modification

As previously mentioned, the unhindered N-terminal tails of histone proteins are subject to various post-translational covalent modifications, such as acetylation, methylation, phosphorylation, and ubiquitylation [3-5,7-9]. Many conserved enzymes are involved in adding and removing histone modifications in response to varying signals and stimuli. These modifications, along with the enzymes responsible for "writing" and "erasing," or adding and removing them respectively, make up what is known as the histone code [8,9].

Post-translational histone modifications have been shown to impact gene expression, alter chromatin structure, and recruit additional remodeling enzymes to perform various regulating functions [8,9]. Fascinatingly, many of the functions of histone modifications are conserved as well. For example, chromatin associated with actively expressed genes often contains hyper-acetylated histones and is methylated at lysine 4 (K4) on the N-terminal tail of histone H3. Conversely, tri-methylation at the lysine 9 residue of histone H3 results in transcriptional silencing and heterochromatin formation [10]. Although many histone modifications have been identified, the field of epigenetics is rapidly developing and there is much to be learned about how the histone code impacts genome maintenance.

Heterochromatin Formation in *N. crassa*

DIM-5 is a key chromatin regulator that is required for heterochromatin formation and DNA methylation in *N. crassa* [7,11,12,20-22]. DIM-5 is a member of the lysine

methyltransferase 1 (KMT1) family of histone methyltransferases. KMT1 enzymes are conserved in plants, animals and some fungi [12,20]. This family of enzymes establishes transcriptionally silent heterochromatin domains through post-translational methylation of the lysine-9 (K9) residue on the N-terminal tail of histone H3 [12,21]. DIM-5 methylates H3K9 as part of a five-component protein complex known as DCDC. DCDC is comprised of DIM-5, DIM-7, DIM-9, Cullin4, and DIM-8/DDB1 (DNA damaged binding protein) [11] and is believed to methylate H3K9 via a two-step mechanism (Figure 1). In this proposed mechanism, DIM-5 is first recruited by DIM-7 to form DCDC, and the complex targets A:T-rich regions of chromatin for methylation. Then DIM-5 tri-methylates H3K9 (H3K9me3) in a CUL4/DDB1^{DIM-9}-dependent manner [11].

Methylation of H3K9 by DIM-5 is also required for DNA methylation in *N. crassa.* Tri-methylation of H3K9 (H3K9me3) recruits a complex of Heterochromatin protein 1 (HP1)/ DIM-2 DNA methyltransferase to heterochromatic foci. Once this localization takes place, the DIM-2 DNA methyltransferase methylates cytosine residues and effectively silences transposable elements. In *N. crassa*, 1.5% of cytosine residues are methylated [12].

Significantly, HP1 has been shown to bind methylated H3K9 ~~i~~n vitro [22]. Furthermore, disruption of the activity of either DIM-5 or HP1 results in a complete loss of DNA methylation [21,22]. This indicates that DNA methylation is dependent upon the catalytic activity of DIM-5 and the localization of the HP1 linker protein to heterochromatic foci.

DIM-5 and "Genome Maintenance"

Documented growth defects of *Δdim-5 N. crassa* strains and reports of "genome instability" in multiple KMT1 mutants [1,12,13,23] suggest that DIM-5 and its homologues are critical for genome maintenance. Throughout this proposal, I will broadly define the processes of DNA replication, DNA repair, and chromosome segregation as *genome maintenance,* and I will use the term *genome instability* to describe defects in these processes. **The central hypothesis of my research project is that DIM-5 is required for proper DNA replication. This hypothesis is supported by data from studies of *N. crassa* mutants lacking DIM-5 and from genetic studies of DIM-5 homologs.**

The phenotypic growth defect of *Δdim-5* in *N. crassa* has been characterized as slow and irregular. Measurement of apical growth of wild type and *Δdim-5* strains under normal growth conditions revealed that the *Δdim-5* mutant grows at nearly half of the rate of the wild-type strain on minimal medium (4.9 ± 0.1 and 2.4 ± 0.7 mm h-1, respectively [12]). Additionally, *Δdim-5* colonies produce fewer conidiophores, and while wild-type *N. crassa* mycelium exhibit a characteristic orange color, *Δdim-5* strains often appear lighter orange to white in color.

Furthermore*, Neurospora* is not the only organism to exhibit defects in growth and development when the KMT1 homologue is impaired, demonstrating that there is, indeed, a conserved relationship between DIM-5 and genome stability. The homologue responsible for mono- and tri-methylation of H3K9 in the pathogenic mold *Aspergillus fumigatus* is ClrD. Studies have shown that disruption of this methyltransferase results in several growth abnormalities including radial growth defects, reduced conidial

production and delayed conidiation [23]. Deletion of the KMT1 homologue, Clr4, in the filamentous yeast *S. pombe* results in diminished growth, de-repression of silent mating-type loci and several abnormal chromosomal issues [23]; and removal of the H3K9 methyltransferase, Suv39h, in mice causes severely impaired viability [13].

Increased Sensitivity to MMS in *Δdim-5*

Fascinatingly, several additional pieces of evidence suggest that DIM-5 is specifically involved in the regulation of DNA replication. Methyl methanesulfonate, MMS, is a DNA damaging agent that causes aberrant alkylation of purine and pyrimidine rings [11,24]. More exactly, MMS modifies adenine to 3-methyladenine, which blocks replication from progressing, and it modifies guanine to 7-methylguanine, which causes base mispairing but does not block DNA replication [24]. Importantly, *N. crassa dim-5* mutants exhibit an increased sensitivity to MMS, suggesting that DIM-5 is involved in regulating DNA replication or repair.

γH2A Localization in *Δdim-5*

Supporting these data is the recent work involving γH2A enrichment levels in *N. crassa.* γH2A is a well-characterized and conserved marker of genotoxic stress [25]. In response to DNA damage and double-strand breaks (DSB), ATM or ATR kinase phosphorylate the C-terminal serine residue of the core histone H2A. Notably, mammals contain a specific variant of H2A known as H2A.X, and it is this variant that is phosphorylated to produce γH2A. Although *Neurospora* lacks this specific histone variant, it contains an H2A.X-like sequence on histone H2A that is similarly modified in response to genotoxic stress. It is therefore possible to locate DNA damage sites based on γH2A localization. Upon localization, γH2A subsequently recruits numerous chromatin-

binding proteins to the sites of DNA damage and helps to recover genome stability by stabilizing stalled replication forks and facilitating DSB repair [25].

In wild-type *N. crassa,* γH2A is enriched in heterochromatin regions. However, Δ*dim-5* strains exhibit globally elevated γH2A levels, indicating that DNA damage is globally elevated when the function of DIM-5 is disrupted [25]. Once again, these data suggest that DIM-5 is involved either in regulating faithful DNA replication or repair. However, Δ*dim-5* strains are not hypersensitive to double-strand break inducing agents, such as ionizing radiation or bleomycin, meaning Δ*dim-5* strains are no less able to repair double-strand breaks than wild-type strains. Together, these data clearly suggest that DIM-5 in involved in the promotion of faithful DNA replication.

The Cell Cycle

The well-defined cell cycle provides a model for the chronology by which the duplication and transmission of genetic material take place. In the simplest of terms, the cell cycle can be divided into four distinct phases, G1, S, G2 and M. The G1 and G2 phases of the cell cycle represent two "gaps" between the landmarks of DNA synthesis (S phase) and mitosis (M phase) [26]. During G1, the first gap, cells prepare for DNA synthesis by duplicating cellular contents. S phase follows, during which time the entire genome is replicated one time per cell cycle. During the second gap, G2, DNA replication errors are repaired as the cell prepares for mitosis. Finally, the cell cycle culminates in mitosis, M phase, followed by cell division resulting in the production of two daughter cells [26].

Although, this sounds simple enough, it has become clear in more recent years that genome stability of eukaryotes is dependent upon tightly regulated and relatively

predictable replication kinetics [27]. In other words, there exists a timeline for replication of varying segments of the genome that often correlates with gene expression and chromatin structure [5,27]. For example, gene dense euchromatic regions typically correlate with early S phase replication while most repeat and A:T-rich regions associated with heterochromatin are characteristically replicated late in S phase [5,12,27,28]. Known exceptions include the heterochromatic pericentric region and *matK* locus of *S. pombe*, which both replicate early in S phase [29].

DNA Replication Control

In order for genetic information to be effectively transmitted, accurate DNA replication must take place in all chromatin environments. This means that all DNA must be replicated accurately and only one time per cell cycle. However, the repetitive DNA sequences in heterochromatin domains pose challenges to genome integrity during DNA replication and repair for several reasons. These sequences can adopt non-B-form DNA structures that stall replication forks and are common sites of illegitimate recombination [25]. This, coupled with the complexity of the replication kinetics in heterochromatic regions, suggests that complex regulation of replication must take place to ensure that efficient and accurate DNA replication is completed.

DNA replication is semi-conservative, anti-parallel and complimentary in nature. The process proceeds bidirectionally to produce two final copies of DNA in which one strand of each copy is of parental origin and the other strand of each copy is a daughter strand, newly synthesized in the 5'-3' direction [30,31]. In the simplest of terms, DNA replication can be divided into three phases, initiation, elongation and termination. This integral process to the proliferation of life is anything but simple, but a review of the

phases of DNA replication will aid in the understanding of some key players and targets of replication control [30,31].

DNA Replication Initiation

Initiation is the phase of DNA replication that is arguably the most susceptible to epigenetic control. Regulation of where in the genome replication initiates, when replication occurs and how initiation transpires must all be effectively controlled in order for a given genome to be effectively duplicated. Furthermore, a number of studies have indicated involvement of chromatin structure and histone modifications in both site localization and replication kinetics [32,33]. Therefore, specific attention will be given to the details of replication initiation in an attempt to predict the role of DIM-5 in the promotion of faithful DNA replication in *N. crassa*.

Origin Characterization

Initiation of DNA replication begins at locations on the genome referred to as origins of replication [34-39]. Prokaryotes typically contain a single origin of replication called the *oriC*, which is located adjacent to the gene encoding the conserved bacterial initiator protein, DnaA [35]. Because of this conservation of symmetry, over 1,300 bacterial origins of replication have been identified despite the fact that the sequence and length of the origins themselves vary amongst bacterial species [35].

Unlike bacteria, eukaryotic organisms contain multiple origins of replication, which often fire asynchronously [36]. Eukaryotic origins associate with hexameric initiator complexes known as the origin recognition complex (ORC). Although the sequences and structure of the ORC may vary amongst eukaryotes, the presence and

function of ORC as the single initiator complex of DNA replication is a highly conserved eukaryotic feature [37].

S. cerevisiae origins are uniquely specific and predictable. They were first identified using an autonomously replicating sequence assay, which assessed the ability of given DNA fragments to initiate autonomous replication in a bacterial plasmid with yeast background [38,39]. Approximately 12,000 autonomously replicating sequences (ARS) have been identified to date, and each of them contains an 11 bp or 17 bp ARS consensus sequence (ACS) that is capable of binding the ORC in preparation of replication initiation [32,38,39]. However, only about 400 (3.3%) of the identified ARS in *S. cerevisiae* have actually been classified as functional [38,39]

Origins of replication in the fission yeast *S. pombe* are not as easily identifiable as the *oriC* of prokaryotes or the ARS of *S. cerevisiae*. This is because they do not display the same kind of predictable sequence specificity. In fact, although many studies have been conducted in search of autonomously replicating consensus sequences in eukaryotes, the properties of the *S. cerevisiae* ACS have remained entirely unique, and nothing similar has been identified in any other eukaryote to date [37]. However, *S. pombe* origins are characterized by A:T-rich islands that are recognized and bound by the A:T-hook domains of ORC4 [32]. Additionally, conduction of single molecule deep sequencing of replication origins, as well as genome-wide nucleosome occupancy tests, in *S. pombe* and *Schizosaccharomyces octosporus* (a second fission yeast species) revealed that polyA tracts and nucleosome exclusion are strong predictors of origin function, along with A:T-richness [39].

The origins of replication of metazoans are even less well defined, however, they do share a number of DNA sequence motifs that may facilitate ORC recognition and binding. Some of these motifs include A:T-rich islands, asymmetrical purine:pyrimidine sequences, dinucleotide repeats and matrix attachment region (MAR) sequences [32,33]. Additionally, it is apparent that the topology of DNA facilitates ORC selection in some organisms. For example, ORC seems to exhibit a preference for supercoiled DNA in *D. melanogaster*, and in humans there have been reports of the association of topoisomerases with replication origins [32].

Origin Number and Nucleotide Length

Variations in origin number and nucleotide length confer a primary level of DNA replication efficiency in eukaryotes. The number of origins of replication in a given genome varies widely and depends largely on chromosome size and genome complexity [35,38]. Typically, larger genomes require a greater number of origins to ensure that replication takes place in a timely manner. Therefore, while prokaryotes contain a single origin of replication, yeast contain up to 400 origins of replication, and humans 30,000-50,000 origins of replication [35]. The nucleotide length of replication origins also increases with genome size and complexity. For example, prokaryotic origins range from 100-1,000 bp in length [35], *S. cerevisiae* origins are approximately 150 bp in size, *S. pombe* origins range from 500-1500 bp and metazoan origins have been shown to range in size from 2 kb – 55 kb sometimes earning the label of "broad initiation zones"[38].

Origin Activation

Despite the variation in origin size and composition, origin function remains highly conserved in eukaryotes. However, in order for the origins of replication in a given

genome to fire and thereby initiate DNA replication, they must first be activated. Activation of eukaryotic origins is a multi-step process that begins with origin "licensing." Licensing involves the recruitment of initiator and replicative proteins, followed by the assembly of a pre-replicative complex (pre-RC) at the site of replication. Eukaryotes primarily depend on the hexameric initiator complex, ORC, to recruit conserved elements of the pre-RC to the origin. Cdc6p is recruited first in order to increase the binding stability of ORC to origin DNA [34]. The association of Cdc6p with ORC also links cell cycle control to origin activation. ORC and Cdc6 work together to control the frequency of DNA replication in relation to cell cycle progression [38,40]. To that end, recruitment and assembly of the pre-RC takes place at the end of mitosis/beginning of G1 phase of the cell cycle [37-40]. Cdc6 then acts as a loading factor for the replicative helicase complex (MCM) [40].

Next, ORC recruits the mini-chromosome maintenance complex (Mcm2-Mcm7) to replication start sites. Mcm2-Mcm7 functions as the replicative helicase responsible for unwinding the DNA of eukaryotic origins of replication [38]. This step is essential because it makes template DNA accessible to replication machinery [38]. Loading of Mcm2-Mcm7 is also the final step in origin licensing. Notably, many organisms including *S. pombe, D. melanogaster, and Xenopus laevis* require an additional pre-replicative loading factor, Cdt1, to load Mcm2-Mcm7 at the replication start site [34,38].

Still, a licensed origin is not activated until S phase. During the G1 to S phase transition, there is a notable increase in level and activity of the major cyclin-dependent kinase, Cdc28, together with its regulatory subunits, the B-type cyclins (Clb-Cdc28), and kinases Cdc7/Dbf4 [35,41,42,43]. The increase in kinase activity recruits additional

initiation factors, such as Cdc45, α DNA polymerase and the GINS complex, to the origin of replication [35,42]. Recruitment of these initiation factors triggers MCM helicase activity, and the origin is converted to an actively firing state permitting DNA replication to proceed [42]. Finally, ORC guides remaining replication machinery to the origin of replication, replisomal proteins associate with the single-stranded DNA and new replication forks are formed [35].

Origin Selection

Importantly, the existence of a eukaryotic origin does not necessarily guarantee its firing. Some origins may be activated every cell cycle while others almost never fire. This may be due to differences in cell type, environmental pressures or any number of factors affecting the cell cycle [35, 36]. In fact, it has been estimated that the overall efficiency rate of *S. cerevisiae* and *S. pombe* origins is less than 50% and the efficiency rate of certain metazoan origins of replication is only 5%-20% [39]. Thus, origin activation and selection are key targets of replication control, and understanding why and how a particular origin is selected for licensing and activation would greatly advance our knowledge in this field.

Timing and Replication Kinetics

It has been previously mentioned that genome duplication relies heavily on tightly regulated replication kinetics. This is due to the demand for complete replication of a given genome during S phase of the cell cycle without “re-replication” of any portion of the chromatin. Coordinated movement is therefore essential to the fulfillment of such a complicated task. Although there are some exceptions to this rule, it has been broadly determined that gene-rich euchromatin regions typically replicate early in S phase while

repeat-rich heterochromatin regions replicate late in S phase [5,12,27,28]. In multicellular eukaryotes, approximately 75% of genes replicate in the first half of S phase [27].

Local effects such as histone modification and chromatin composition have been shown to influence pre-RC formation and initiator/replicator protein association at origins of replication supporting a model in which replication timing is the result of competing signals that determine origin activation based on replication factor availability or binding capacity (MCM, Cdc45, Sld2, Sld3, DDK) [27]. However, evidence suggests that the size of a genome influences the degree to which replication timing will be affected by such local factors. While smaller genomes are more directly influenced by local regulation, larger genomes are more predominately influenced by large-scale chromosome folding, or 3D chromatin organization, and less likely to be affected by localized effects [27].

Epigenetic Examples of Replication Control

To date, a number of studies have implicated multiple histone modifications in the regulation of DNA replication. Histone lysine acetylation has been linked to the regulation of replication timing and has been shown to facilitate replication at certain initiation sites of yeast, *D. melanogaster* and *Xenopus* egg extracts [32,35,42]. However, of particular interest are the correlations between histone lysine methylation and DNA replication.

H3K36 Methylation

Genome-wide studies have revealed that the methylation state of H3 lysine 36 (H3K36) results in two opposing effects on replication timing in *S. cerevisiae* [42,44]. H3K36 methylation regulates the time of association of the initiation factor Cdc45 with origins of replication and, therefore, impacts the timing of origin firing [42,44]. Tri-

methylation of H3K36 (H3K36me3), in conjunction with histone deacetylation, delays the time of Cdc45 association and results in late firing origins of replication. However, mono-methylation of H3K36 (H3K36me1), in conjunction with histone acetylation, accelerates Cdc45 association and results in early firing origins of replication. Furthermore, deletion of the SET2 methylatransferase responsible for methylating H3K36 depletes all forms of H3K36 methylation and delays Cdc45 association in a manner that is completely independent of histone acetylation state [42,43].

A current model for how H3K36me3 mediates this inhibitory effect on origin firing depends on the mutual association of protein Eaf3 (a component of the histone acetyltransferase complex NuA4 [45]) with H3K36me3 and histone deacetylase, Rpd3 [42]. It has been postulated that H3K36me3 inhibits origin firing through Eaf3-mediated recruitment of Rpd3, causing histone deacetylation and decreased chromatin accessibility [42]. Results from a number of experimental techniques have produced evidence in support of this model [42]. Since changes in the methylation state of H3K36 have opposing effects on the timing of origin firing, this particular histone modification is clearly important to the regulation of replication kinetics in *S. cerevisiae* [42,44]

H4K20 Methylation

In humans, mono-methylation of H4K20 promotes pre-RC assembly at given loci [42]. Studies have shown that tethering Set8(PR-Set7), the methyltransferase responsible for methylating H4K20, to a given locus will promote pre-RC assembly at that site [35,42]. Additionally, H4K20me1 levels are high at points during the cell cycle when pre-RCs are being formed (M and G1 phases) and low during S phase [35,42], and deletion of Set8(PR-Set7) causes cell cycle arrest [42]. Although the exact mechanism by

which H4K20me1 is involved in the recruitment of pre-RC components is unknown, it can be concluded that H4K20me1 plays a role in origin selection and activation and an indirect role in replication kinetics. Fascinatingly, aberrant persistence of Set8(PR-Set7) through S phase results in extensive genome re-replication, while di-methylation of H4K20 (H4K20me2) in S phase appears to inhibit re-licensing of origins once they have fired [42]. This suggests yet another case for competing functions of opposing methylation states, supporting the model in which replication timing is the result of competing signals that determine origin activation based on replication factor availability or binding capacity [27,42].

H3K4 Methylation

It is worth noting that H3K4me2/3 enrichment correlates with early replicating origins of human cells. Conversely, late replicating regions of the genome show depletion of H3K4me2/3, and similar enrichment patterns of H3K4me2/3 have been seen in *D. melanogaster* [42]. Less is known about the function of this particular post-translational histone modification; however, it is currently thought to be involved in the regulation of origin firing [42]

H3K9 Methylation

Deletion of the H3K9 histone methyltransferase G9a is a lethal mutation in mice [46]. Deletion of G9a in mouse embryonic stem cells (EBS) results in significant reduction of H3K9 di-methylaiton (H3K9me2) and significant up-regulation of 167 genes [46]. However, no change in replication timing is detected in the mutant. The majority of G9a-repressed genes normally replicate late in S phase, and they continue to replicate late in S phase when G9a is deleted [46]. However, mammals have four H3K9

methyltransferase enzymes; therefore, deletion of only G9a does not indicate a complete loss of H3K9 methylation. Furthermore, since G9a seems to primarily mediate H3K9 di-methylation, the possibility remains that another mammalian H3K9 methyltransferase is responsible for mediating replication timing through the regulation of replication factor availability or binding.

Extrapolating from the data above regarding the known involvement of histone methylation in replication control, it seems possible that H3K9me3 promotes faithful DNA replication via regulation of replication kinetics. Perhaps H3K9 methylation regulates the access or binding capacity of yet another replication factor. If this were true, removal of H3K9me3 could result in disturbance of normal replication kinetics, meaning that heterochromatin regions that are typically replicated late in S phase could be replicating early in S phase and causing the phenotypic growth defect observed in Δ*dim-5* strains [12].

HP1 and DNA Replication Control

The relationship between the H3K9 methyltransferase and HP1 provides further support for the role of DIM-5 in the promotion of DNA replication. HP1, bound to methylated H3K9, has been shown to serve as a platform for the recruitment of various effectors involved in transcriptional silencing, modification of histones, chromosome segregation and even DNA replication [29]. The *S. pombe* HP1 homologue, Swi6, is required for initiation of replication at the heterochromatic loci of pericentromeric regions and the silent mating type locus, *matK* [29]. Swi6 stimulates loading of Sld3 on origins of pericentromeric regions and the *matK* locus [29]. Deletion of Swi6 disrupts the temporal regulation of replication at these regions, resulting in aberrant late S-phase

replication of *matK* and pericentromeric regions, as well as diminished growth [29]. However, if DIM-5 is absent, HP1 cannot bind, and if HP1 cannot bind, Sld3 will not be loaded at the origin. Therefore, deletion of DIM-5 would presumably have the same effects on the timing of DNA replication in pericentromeric regions and *matK* of *S. pombe*, further supporting to the idea that H3K9me3 affects the kinetics of DNA replication.

DNA Replication Elongation

Once replication machinery is in place, the replication fork has been formed, and an origin has fired, DNA chain elongation can begin. The biochemical reaction of chain elongation is as follows [31]:

DNA polymerase

Primer (parental) DNA + {dNTPs} ----------> progeny DNA

Elongation begins with the synthesis of short RNA primers at initiation start sites. These primers are complimentary to the newly exposed single-stranded template DNA and are synthesized by an enzyme called primase [47]. RNA primers are critical to the initiation of chain elongation because DNA polymerase is only able to catalyze the addition of nucleotides to 3' ends of preexisting strands.

Next, DNA polymerase catalyzes the addition of free dNTPs to the 3' hydroxyl group of the growing progeny chain [30]. The DNA product of replication is always synthesized in the 5'-3' direction, and the template strand is read in the 3'-5' direction. Therefore, due to the antiparallel nature of DNA, continuous replication will only be possible for one of the two strands of progeny DNA. The strand that exhibits continuous

replication is called the leading strand, and it is synthesized in the same direction of replication fork movement [31]. The other strand must be synthesized discontinuously, in the opposite direction of replication fork movement. This strand is called the lagging strand. Thus DNA synthesis in the 5'-3' direction of the lagging strand produces short discontinuous segments called Okazaki fragments [31]. Each Okazaki fragment is RNA-primed and typically 100-200 nucleotides in length. RNA primers will eventually be removed and replaced with DNA through the exonuclease and repair activity of polymerase enzymes. Newly synthesized strands of DNA are referred to as nascent strands [47].

DNA Replication Termination

Termination of DNA replication typically occurs when the replisomes, or replication forks, of two adjacent origins converge [48]. Once this contact has been made, the any remaining gaps of DNA in the daughter strands must be filled, and the nascent strands from the adjacent forks must be ligated, or joined together. This ligation is accomplished by an enzyme called ligase. Finally, any remaining DNA helix intertwinings are removed in a process called decatenation, and the replisome is disassembled. Two identical strands of DNA are produced, and replication is complete.

Objective

This knowledge about the H3K9 histone methyltransferase DIM-5, the behavior of *Δdim-5* mutants, and the reported data of the involvement of various DIM-5 homologues in the regulation of DNA replication inspired the following objective. **The objective of this work was to develop new tools to monitor DNA replication in both**

wild-type and *Δdim-5 N. crassa* cells in an attempt to examine the validity of the hypothesis that DIM-5 impacts genome stability at the DNA replication level.

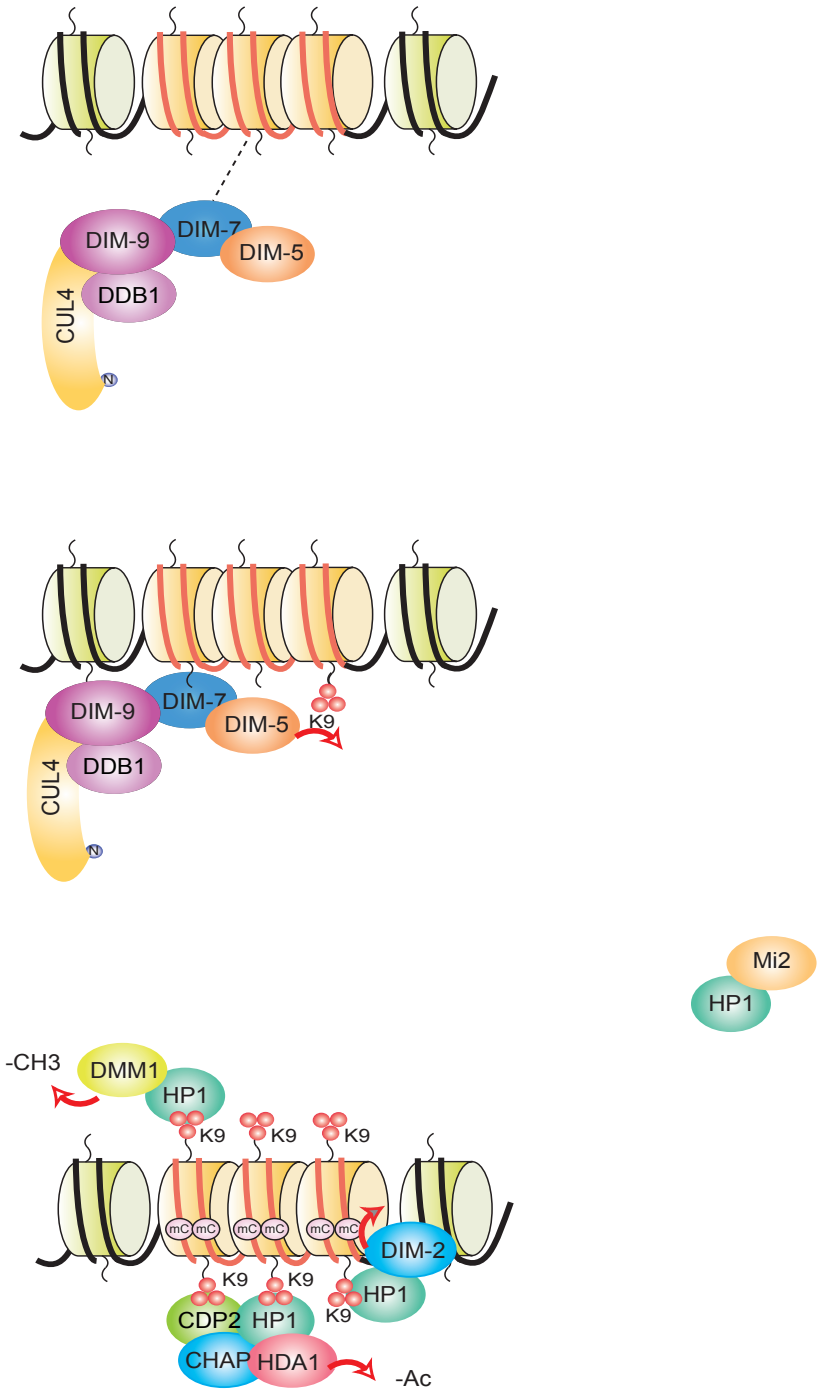

Figure 1: A model for heterochromatin formation in *N. crassa.* DIM-7 recruits histone methyltransferase DIM-5 to A:T-rich DNA (shown in red), DCDC is formed, and DIM-5 tri-methylates H3K9. HP1 then recognizes H3K9me3 and recruits DNA methyltransferase DIM-2. DIM-2 methylates cytosine residues of DNA.

CHAPTER 3

MATERIALS AND METHODS

Isolation and Sequencing of Nascent Leading Strand DNA molecules

I first planned to isolate small nascent leading DNA strands (SNS) across the genomes of WT and *dim-5 N. crassa* strains in an attempt to identify origins of replication (Figure 2). The isolation assay broadly depended on two key selectable features of SNS. According to established methods these nascent molecules can be selected based on predictable size (0.5-2.5KB) and the presence of an RNA-primer at the 5'-end of each nascent strand molecule [47]. It should be noted that all steps prior to lambda exonuclease treatment were conducted under RNase-free conditions in order to protect the RNA primers of SNS molecules from premature degradation.

Strains and Culture Conditions

Wild-type *N. crassa* strain (74-OR23-1VA; FGSC# 2489) was obtained from the Fungal Genetics Stock Center [49]. A *dim-5* knockout strain, (S132; N3074) *Δdim-5::bar+*, was created previously by replacing the *dim-5* gene with a basta-resistance cassette [50,51]. I will refer to this knockout strain as *dim-5.* Cultures of each strain were inoculated in 50 ml of solid Vogel's minimal medium (VMM) in 250 ml flasks then grown at 32°C for five days.

Once sufficient growth density was achieved, 10-15ml of liquid VMM was added to each flack. Flasks were vortexed to mix, and mycelia were separated from the conidial suspension of each sample via cheesecloth filtration. Next, the concentration of conidia in

each sample suspension was estimated using a hemocytometer. The volume of each starter culture needed to produce a final concentration of 1x10^6 conidia per ml in a final volume of 1 L was calculated. Then this calculated volume of each starter culture was transferred to 1 L of 1X Vogel's + 1.5% sucrose liquid medium and grown at 25°C for 12-16 hours with shaking (150 rpm). This protocol was developed in order to ensure that cells were harvested during logarithmic growth thereby optimizing the fraction of cells in S-phase at the time of harvest.

Cell Harvest and Cell Wall Digestion

Cells were harvested under RNase-free conditions. Each 1 L sample was filtered using Buchner funnel and filter paper. Next, the samples were washed with 100 mM Tris (pH 9.5) and 10 mM dithiothreitol three times and resuspended in 10-20 ml (depending on culture size) of 1 M sorbitol. Cell wall digestion was completed using a concentration of 0.5 mg of lyticase per liter of original culture and incubated at 32°C with gentle shaking for 1-4 hrs, or until cell walls were fully digested (generously provided by Vincent Starai, UGA).

Cell Lysis

Cell lysis was completed using 2× SDS lysis buffer (1% SDS, 20 mM Tris·Cl, 10 mM EDTA, and 600 mM NaCl*).* Proteinase K was added at a concentration of 200 µg/ml, and cultures were incubated at 37°C overnight.

Genomic DNA Extraction

A relatively large-scale, two-step genomic DNA extraction of lysed cells was completed in an RNase-Free environment using Phenol/Chloroform (pH 8.0) followed by chloroform as described (protocol A, using 2X SDS Buffer) [47].

Separation of DNA Fragments According to Size via Sucrose Gradient Centrifugation

A 5-30% sucrose gradient was prepared by hand. First, 18 ml 5% sucrose was pipetted into Beckman SW28 tubes. Then 17 ml of 30% sucrose was slowly added underneath the 5% sucrose layer using syringe. Samples were heated to generate single stranded DNA molecules and then loaded on top of each corresponding sucrose gradient. Next, gradients were placed in an SW40 swinging-bucket rotor, and DNA was separated according to fragment size as previously described [47].

Selection of Gradient Fractions Containing Short Nascent DNA Strands (SNS)

Samples were harvested in 36 fractions by pipetting 1ml at a time from the top of each sucrose gradient. Pipette tips were cut in order to prevent DNA breakage during harvest. According to established methods, contaminating Okazaki fragments were expected to be ~100-200 bp in size and to migrate to the top of each sucrose gradient. Therefore, to separate Okazaki fragments from SNS, only DNA that was 0.5-2.5KB in size was considered in this assay. According to updated sources, SNS of this size were expected to have migrated between fractions 16 and 23 of the sucrose gradient [47]. Fractions were pooled and precipitated overnight as described in order to concentrate SNS [47].

To determine the size of the collected DNA fragments, fractions were visualized via agarose gel electrophoresis at a neutral pH [47]. Fractions containing DNA of the appropriate size (0.5-2.5 KB) were identified and selected in an attempt to isolate SNS based on expected short nascent DNA strand size [47].

T4 Kinase Treatment and Lambda Exonuclease Digestion

Next the selected fractions were treated with T4 polynucleotide kinase as described to phosphorylate any contaminating single-stranded DNA that was non-SNS DNA [47]. Lambda exonuclease preferentially digests both double-stranded DNA and phosphorylated single-stranded DNA, but will not degrade SNS molecules due to the presence of the 5' RNA primer on nascent fragments. Following treatment with T4 polynucleotide kinase, samples were treated with lambda exonuclease under the described conditions [47].

Second-strand synthesis and Sequencing

After exonuclease treatment, samples were treated with RNase in order to remove the remaining RNA primers on each strand. Samples were purified using a Sera (Ampure) bead cleanup at a 1:1 DNA to bead concentration. Then the resulting DNA fragments were treated with Random Primer Solution and Klenow as described [47] to produce a double-stranded DNA product. DNA was purified using a QIAquick PCR purification kit. Finally, samples were prepared for sequencing using an Illumina TruSeq kit (Illumina cat-FC-121-2002). Libraries were prepared according to manufacturer instructions, and PCR was performed for 12 cycles. Illumina sequencing was performed at the University of Georgia Genomics Facility using an Illumina NextSeq 500 instrument.

Sequence reads were mapped to the latest *N. crassa* genome annotation (version 12), available from the *Neurospora* genome database [14], using bowtie2 [52]. The Integrated Genome Viewer (IGV), available from the Broad Institute website [53,54] was used, as described, to visualize read density [25], and igvtools was used to count read

numbers for 25-bp bins. Additionally, a custom feature annotation file, previously created in IGV, was used to calculate normalized enrichment values [25].

Fluorescent Reporter Construct Development

In an effort to develop a new tool to monitor the time of S-phase in *N. crassa,* I introduced a nuclear-localized, photoconvertable fluorescent Dendra2 reporter construct to the *N. crassa* genome (Jones K, Kim DW, Zhu J, Kang E, Momany M, Hagen A, Lewis Z, Khang CH., unpublished). When exposed to 405 nm light, this emission wavelength of the protein is converted from green to red. The conversion is irreversible; therefore, isolation and photoconversion of a single nucleus would create a novel approach for monitoring the timing of cell cycle progression in *N. crassa*.

Strains and Growth Conditions

Wild-type *N. crassa* strain (74-OR23-1VA; FGSC# 2489) was obtained from the Fungal Genetics Stock Center [49] and grown as described on Vogel's Minimal Medium (VMM) [15]. To introduce the H1-Dendra2 construct into *Neurospora*, pCK1285 was used as a template for PCR, and primers #216 (AACACATTGCGGACGTTTTT) and #217 (TTTAAACTGAAGGCGGGAAA) were used to generate a linear fragment containing the *hph*+ gene driven by the TrpC promoter and the hH1-dDendra2 fusion driven by the *Magnaporthe oryzae* RP27 promoter (Figure 3).

Transformation of N. crassa/pCK1285

The H1-Dendra2 construct was transformed into wild-type *N. crassa* (strain 74-OR23-1VA; FGSC# 2489) [49] using established methods [55]. Transformants were selected on Vogel's Minimal Medium supplemented with 200 ug/ml hygromycin B (Invivogen).

Visualization and Photoconversion of N. crassa

Epifluorescence microscopy was performed using an LSM 510 confocal microscope system equipped with 405 nm, 488 nm, and 543 nm lasers. For pre-conversion imaging, the Argon (488 nm) laser at 5% power and emission spectra of 505-530 was used for green fluorescence. For post-conversion imaging, the 543nm laser at 80% power and emission spectra of 560-615 was used for red fluorescence. Zen Black (Carl Zeiss) software was used for image capture and processing.

Photoconversion of *N. crassa* nuclei was accomplished by irradiating the selected nucleus with the 405 nm laser at 100% power and a zoom factor of 2x and pixel dwell time of 2.56 µs. Samples were exposed to 405 nm irradiation for approximately 10 seconds. Any longer exposure produced undesired photo-bleaching effects. 200 iterations were taken for all imaging.

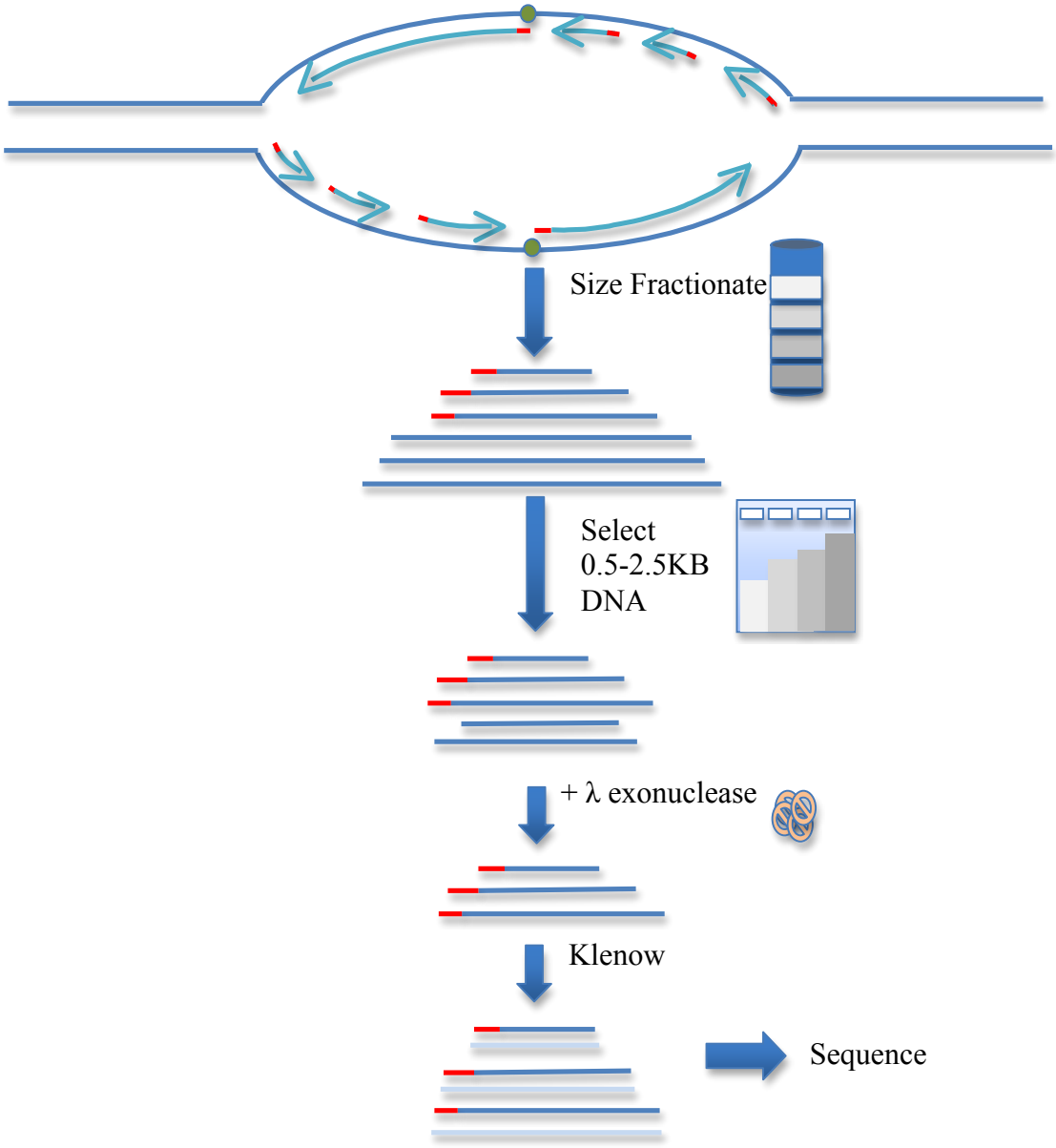

Figure 2: Schematic of short nascent DNA strand (SNS) abundance assay. DNA is isolated from cultures in logarithmic growth then size fractionated via sucrose gradient centrifugation. Fragments 0-5-2.5 KB are selected using gel electrophoresis as a guide. This ensures removal of contaminating Okazaki fragments. Fragments 0.5-2.5 KB in size are then treated with lambda exonuclease to remove all remaining contaminating non-SNS DNA Lambda exonuclease digests all DNA that is not RNA primed. The isolated SNS DNA is then purified and prepared for sequencing. This involves second strand sequencing and Illumina library preparation steps. Finally, DNA is submitted for sequencing.

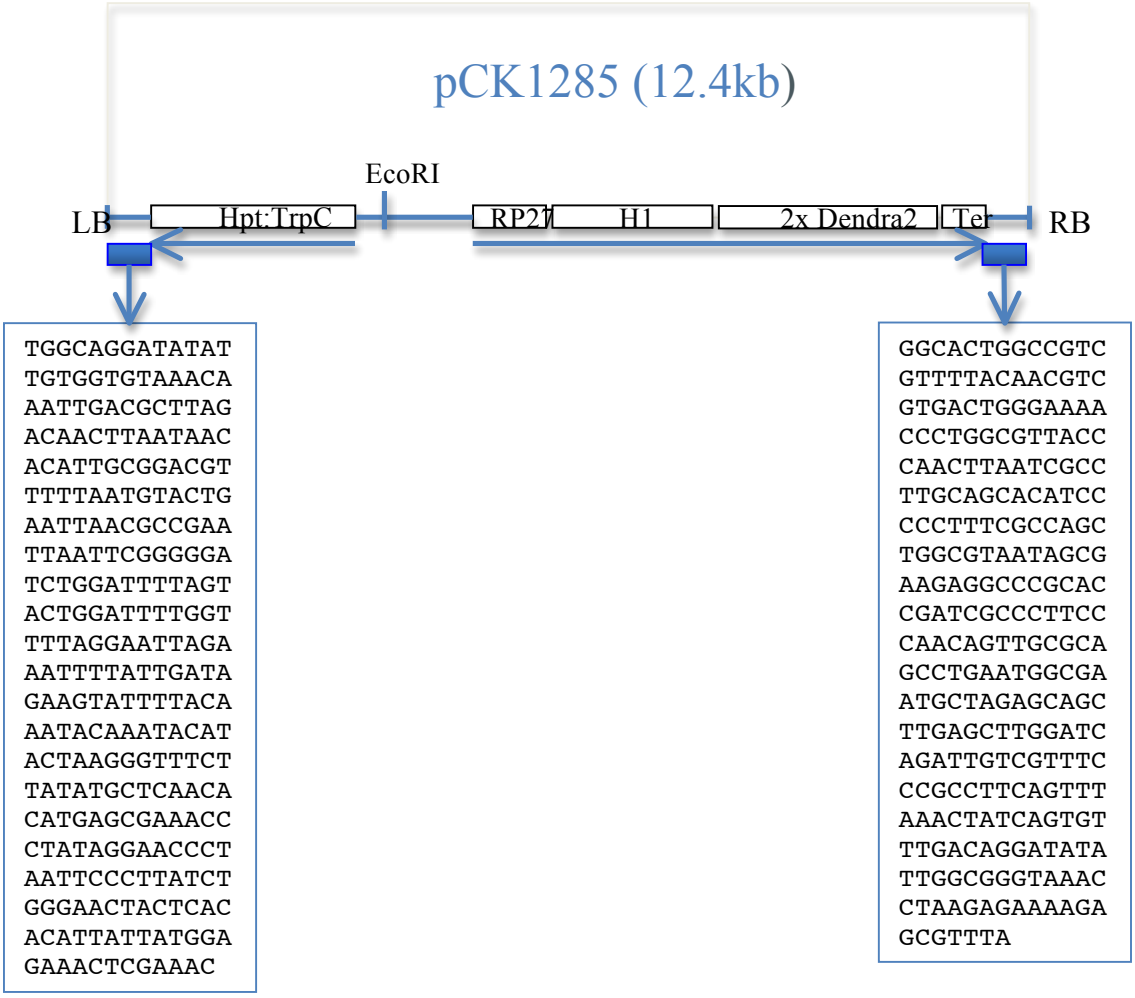

Figure 3: pck1285 plasmid construct. (Courtesy of Chang Hyun Khang, University of Georgia). Primers #216 (AACACATTGCGGACGTTTTT) and #217 (TTTAAACTGAAGGCGGGAAA) were used to generate a linear fragment containing the *hph+* gene driven by the TrpC promoter and the hH1-dDendra2 fusion driven by the *Magnaporthe oryzae* RP27 promoter.

CHAPTER 4

RESULTS

Nascent Strand DNA Sequencing

The goal of this work was to identify origins of replication in the *N. crassa* genome by isolating nascent leading DNA strand molecules across the genome. DNA synthesis proceeds symmetrically and bi-directionally away from replication start sites, meaning that at the center of each nascent leading DNA strand lies an origin of replication. It is important to note that this is not the case for lagging strand DNA. The Okazaki fragments (~100-200 bp in size) resulting from discontinuous DNA replication do not straddle origins of replication as do the anti-parallel counterpart leading nascent strands. Therefore, only leading strand nascent DNA was isolated in this assay.

Key elements to the success of this isolation protocol included proper growth conditions and harvest time, effective size fractionation via sucrose gradient centrifugation, and complete lambda exonuclease digestion of contaminating, non-SNS DNA. Until lambda exonuclease digestion, all steps were conducted under RNase-free conditions in order to protect the RNA primers associated with SNS molecules from premature degradation.

Large batches of asynchronously dividing wild-type and *dim-5* cell cultures were harvested after 12-16 hours of growth at 25°C with shaking (150 rpm). My goal was to optimize the fraction of cells in each culture that were in S-phase (DNA replication) at the time of harvest in order to increase SNS concentrations. Therefore, growth conditions

were designed to promote cell harvest during logarithmic growth. As expected, growth of *dim-5* was noticeably less dense than that of wild-type at the time of harvest.

Next, samples were size fractionated by sucrose gradient centrifugation. This step allowed me to perform a multipurpose selection. Primarily, it facilitated the selection of short nascent strands according to the predicted SNS size of established methods [47]. Secondly, it allowed me to eliminate the contaminating Okazaki fragments found on lagging strands throughout the genome.

To determine the effectiveness of DNA size separation, I visualized the results of wild-type and *dim-5* sucrose gradients using agarose gel electrophoresis under non-denaturing conditions. 1KB plus genomic ladder was used for guidance. Size separation of the DNA fragments was successful for both the wild-type and *dim-5* samples (Figure 4A and 4B). As expected, the smallest DNA fragments of each sample migrated to the top of respective sucrose gradients. These fragments were harvested first and therefore appear in the earlier fractions, loaded in the left lanes of each agarose gel. In contrast, the heaviest fragments of DNA migrated to the bottom of each sucrose gradient and were therefore harvested last. These fragments can be seen at the far right-hand lanes of the agarose gel of each sample.

According to convention, DNA 0.5-2.5 KB in size was selected in search of Short Nascent DNA Strands (SNS) [47]. This size selection ensured the exclusion of Okazaki fragment contaminants, which are expected to be approximately 100-200 nucleotides in length and are located on lagging strands throughout the genome [47]. It also ensured the exclusion of very short leading nascent DNA and/or degraded DNA. However, the selected 0.5-2.5 KB fractions were still contaminated with broken DNA fragments at this

time, and it was therefore necessary to treat the samples with lambda exonuclease in order to truly isolate SNS for sequencing.

Lambda exonuclease only digests DNA from 5'-ends; the enzyme does not exhibit exonuclease activity on RNA. This means that the RNA-primed nascent DNA strands in each sample should remain undigested after lambda exonuclease treatment. A lambda exonuclease activity assay was performed in order to ensure that the exonuclease enzyme was properly digesting double-stranded DNA as well as phosphorylated single-stranded DNA fragments [47]. Digestion of the tested plasmid was complete, indicating efficient lambda exonuclease activity. Therefore, wild-type and *dim-5* samples were also treated with the lambda exonuclease enzyme.

At this point, samples were expected to contain isolated SNS molecules. These were treated with RNase to remove the RNA primers, and second strand synthesis was performed using random primers and Klenow polymerase. After purification and Ilumina library preparation, wild-type samples of concentrations ranging from 15.3 nM-19.8 nM and *dim-5* samples of concentrations 13.0 nM-13.92 nM were submitted for sequencing.

Unfortunately, even though all images and quantitative analysis indicated that size separation and isolation of SNS had been effective via this nascent strand assay, the DNA sequencing results generated by the University of Georgia Genomics Facility using an Illumina Next-Seq500 instrument for both the wild-type and *dim-5* samples did not show any notable peaks in the genome (Figure 5).

Photoconversion of Dendra2 in *N. crassa*

I wanted to know if Dendra2 would be a useful cytological tool in *N. crassa*. I therefore fused an hph selectable marker, encoding hygromycin B resistance, to the hH1-

Dendra2 construct (Generously donated by Chang Hyun Khang, University of Georgia). Then I introduced this hH1-Dendra2-hph fusion construct to *N. crassa* by transformation. I isolated 10 transformants that were selected on VMM/ 200 ug/ml hygromycin B and tested for successful transformation and Dendra2 expression by visualizing green fluorescence using an epifluorescence microscope. All 10 transformants exhibited fluorescence consistent with nuclear localization of the fusion protein. I next tested for photoconversion of Dendra2 in *N. crassa* by exposing a single nucleus to 405nm light using a LSM 510 confocal microscope. A 10 second exposure resulted in the appearance of red fluorescence in all transformants (Figure 6), indicating that Dendra2 can indeed be used in *N. crassa* to track cell cycle and nuclear division.

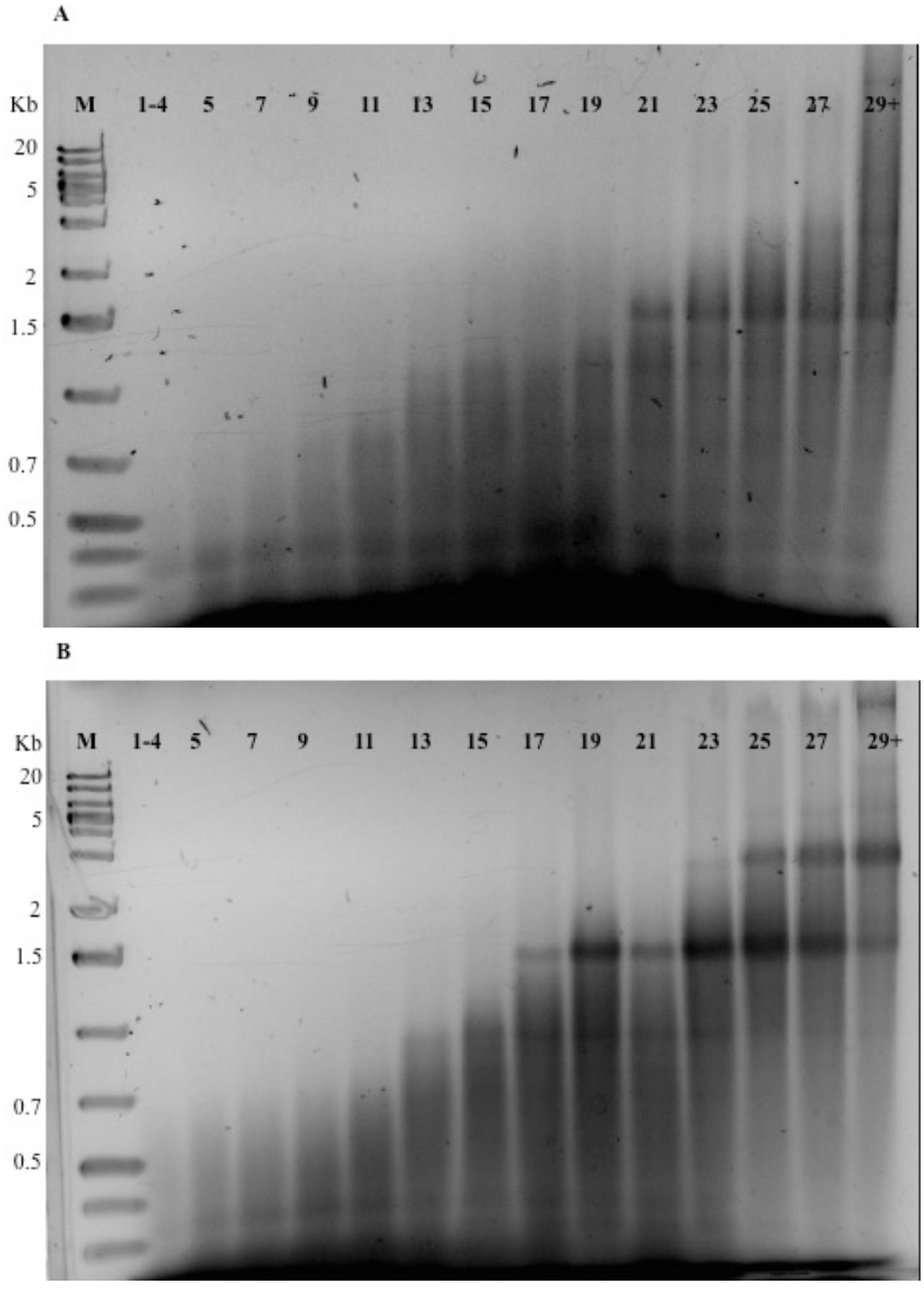

Figure 4: Agarose gel electrophoresis images obtained after sucrose gradient size fractionation. (A) Wild-type *N. crassa* fractionated DNA. (B) *dim-5 N. crassa* fractionated DNA

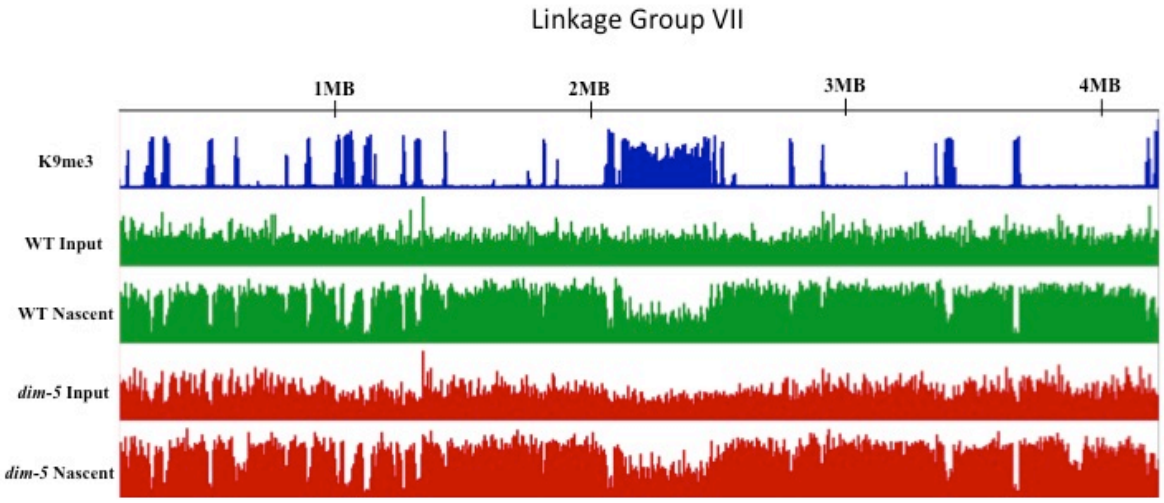

Figure 5: Normalized enrichment values of SNS data across the *N. crassa* chromosome LGVII (Linkage Group VII). Blue track indicates H3K9me3 enrichment across the chromosome. Green tracks indicate the enrichment values of the input and nascent strand isolation results in the Wild-type *N. crassa* strain. Red tracks indicate the enrichment values of the input and nascent strand isolation results in the *dim-5* strain.

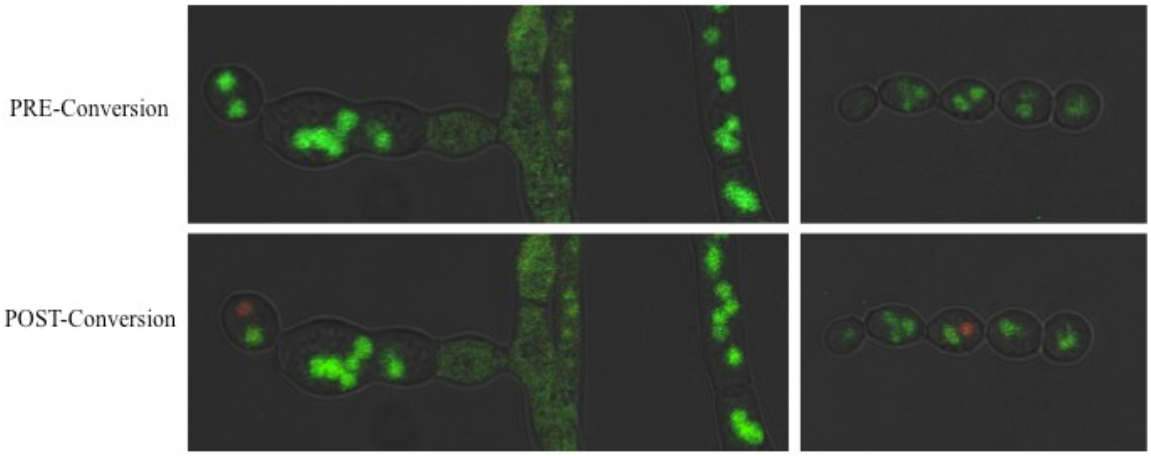

Figure 6: Microscopy images of photoconversion of the Dendra2 fusion construct at single nuclei in *N. crassa*. Top panel of images were captured PRE-conversion with 405nm laser. Bottom panel of images were captures POST-conversion with 405 nm laser.

CHAPTER 5

DISCUSSION

Effective DNA replication must occur in all chromatin environments to ensure faithful transmission of genetic information, and instability resulting from defects in DNA replication is frequently associated with disease including cancer [1,3,4,7,56]. Therefore, a better understanding of the cellular mechanisms that ensure or impair faithful DNA replication may provide key insight into the diagnosis, treatment, and prevention of disease.

Origins of replication are the sites in a given genome where DNA replication is initiated. Proper origin firing is fundamentally essential to faithful DNA replication. Unlike bacteria, eukaryotic organisms contain up to thousands of origins of replication, and origin firing is often asynchronous [36]. The when, where, and how of origin firing across a given genome involves complex signaling and regulation. Furthermore, a number of studies have indicated that epigenetic factors such as chromatin structure and histone modifications help provide the flexible control that such complex regulation requires [1,3,4,32,35,36,42,44].

Identification of Origins of Replication in *N. crassa*

I wanted to identify origins of replication in *N. crassa* because I suspect that the H3K9 methyltransferase, DIM-5, is involved in the promotion of faithful DNA replication. There are examples in multiple organisms of histone modifications impacting the site localization and kinetics of DNA replication [32,33,35,42,44]. I wanted to see if

DIM-5 has a similar role in *N. crassa*, and I recognized that identifying origins of replication in *N. crassa* would provide a useful framework for my study of the epigenetic control of DNA replication. I planned to use a previously established method for mapping origins of replication that involved nascent strand isolation followed by DNA sequencing [47]. Furthermore, because this method only identifies origins of replication from which SNS molecules are actively being replicated, comparison of origin profiles between wild-type and *dim-5 N. crassa* strains could potentially reveal important differences that would shed light on the function of DIM-5 in DNA replication. If no differences were immediately apparent between the two strains, the identification of origins of replication would be a novel discovery in *N. crassa* and would provide a useful foundation for future study of the DIM-5 involvement in DNA replication control.

Unfortunately, the sequence results of this assay did not identify the locations of replication origins across the *N. crassa* genome. No obvious peaks were generated from the sequencing data of the wild-type or *dim-5* strain. However, I believe, for multiple reasons, that I was able to successfully isolate SNS molecules in both wild-type and *dim-5*. First, size fractionation of the DNA fragments of both strains was achieved using sucrose gradient centrifugation (Figure 4). Second, complete activity of the lambda exonuclease enzyme was indicated by the results of the conducted lambda exonuclease efficiency assay. Third, the final concentration of the suspected isolated SNS DNA after RNase treatment, second strand synthesis, purification, and library preparation was adequate for both wild-type and *dim-5* strains.

So if SNS DNA was, in fact, isolated in both wild-type and *dim-5 N. crassa* strains as a result of this nascent strand assay, why did the sequencing results lack

discernable peaks? One of the most likely explanations for these results is a recently discovered PCR bias against A:T-rich DNA sequences [57]. Studies in *N. crassa* have recently concluded that the number of PCR cycles and the polymerase used for PCR amplification can both have an affect on the amplification of DNA, especially in regions that are A:T-rich. In fact, according to these recent reports, A:T-rich DNA was significantly underrepresented after only 8 cycles of PCR using multiple DNA polymerase enzymes [57]. However, a very typical characteristic of origins of replication in eukaryotic organisms is A:T-richness. Therefore, it is likely that significant portions of SNS DNA contain A:T-rich domains. To further complicate matters, the concentration of SNS DNA in a given cell culture is relatively low compared to the combined amount of DNA that is non-nascent and lagging strand nascent. Therefore, PCR amplification at the end of SNS isolation was needed in order to generate quantities of DNA for sequencing.

In the first trial of this experiment, I used 12 cycles of PCR for amplification of the SNS DNA that I had isolated from WT and *dim-5* cultures, but I was not able to generate enough *dim-5* SNS DNA from this trial for sequencing. Since the goal was to optimize the fraction of cells in S-phase at the time of harvest, the phenotypic growth defect of *dim-5* presented a challenge to the experimental design of this assay. It took a bit of troubleshooting in order to plan a growth scheme in which I could harvest both WT and *dim-5* cultures during logarithmic growth and produce high enough concentrations of isolated SNS DNA for sequencing upon completion the assay.

The first thing I did was increase the scale of my work for the second trial of this assay. I also lowered the temperature and increased the growth time to the specifications given in the materials and methods section of this thesis. Investigation of the above-

mentioned PCR bias was being conducted concurrently to my nascent strand work, therefore, the increase in scale of my work allowed me to reduce the number of PCR cycles used to generate my sequencing library for the second trial of this assay. Unfortunately, I still was not able to reduce the number of PCR cycles below the reported 8 cycles bias [57], and the libraries showed no apparent peaks when sequenced.

It is highly possible that the origins of replication in *N. crassa* are A:T-rich and are therefore being underrepresented by the recently documented PCR bias against A:T-rich regions [57]. Still there is room for continued troubleshooting of this assay in the future. Perhaps further increasing the scale of this protocol would allow the number of PCR cycles to be reduced below the documented 8-cycle cutoff for this bias. Perhaps the bias would be less apparent if PCR amplification were conducted with a different polymerase enzyme.

BrdU (5-bromo-2'-deoxxyuridine) Incorporation Assay

Another option for identification of origins of replication in *N. crassa* would be to conduct a BrdU (5-bromo-2'-deoxyuridine) incorporation assay. BrdU is a synthetic analog of thymidine, however fungi lack thymidine kinase, the enzyme that phosphorylates BrdU and potentiates its incorporation into DNA, making this method a bit more challenging in *Neurospora*. In order to conduct BrdU incorporation assays, previously engineered *N. crassa* strains that have functional *Herpes Simplex* thymidine kinase genes incorporated in both wilt-type and Δ*dim-5* backgrounds would have to be utilized [58]. Then DNA could be size fractionated to select SNS DNA. Immunoprecipitation with BrdU-specific antibodies would allow identification of origins

of replication based on the locations of BrdU incorporation at SNS sites across the genome [47].

Chromatin Immunoprecipitation and Sequencing (ChIP-seq) of FLAG-tagged replisome components

It has been revealed through electron microscopy that, when treated with hydroxyurea, S-phase *S. cerevisiae* cells accumulate ssDNA structures that resemble replication bubbles [59]. HU is a known replication inhibitor responsible for dNTP depletion through inhibition of ribonucleotide reductase. In the absence of dNTPs, polymerization slows, and replication eventually arrests. Stalled replication forks are protected from collapse and DSB by a variety of conserved proteins known as the fork protection complex. The *N. crassa* protein Tof1 is a homolog to the core human fork protection protein TIM1[60].

Another potential method for identification of origins of replication in *N. crassa* would be to use optimized concentrations of HU to force stalling of replication forks in both wilt-type and Δ*dim-5* strains containing FLAG-tagged components of DNA polymerase POLD or TOF1 [58,61]. Sites that accumulate stalled replication forks could then be identified through chromatin immunoprecipitation followed by sequence analysis (ChIP-seq). It would be expected that the most frequent stall sites will be at, or adjacent to, origins of replication. Enrichment of Tof1 and PolD at stalled replication forks in HU treated cells could be detected by an anti-FLAG antibody.

Monitoring DNA Replication Kinetics in *N. crassa*

Replication of the entire genome is accomplished during S-phase of the cell cycle, and most eukaryotes appear to replicate heterochromatin at defined times that can be

classified as either early or late in S-phase. In most of the organisms examined to date, heterochromatin is the last portion of the genome to be replicated [5,12,27,28,62], however, *S. pombe* replicates portions of its heterochromatin early in S-phase [28]. The replication timing of *N. crassa* heterochromatin is unknown, and the control of replication timing is overall poorly understood. Little is known about the rate of DNA replication in different chromatin environments of more complex eukaryotes. However, it seems plausible that the tight packaging of DNA might result in slower progression of replication in heterochromatin regions than in the more loosely packaged regions of euchromatin. Since it is known that DIM-5 is involved in heterochromatin formation, determining the timing and rate of heterochromatin replication in both wild-type and Δ*dim-5* may prove valuable in understanding the suspected role of DIM-5 in the DNA replication process.

I wanted to know if the photoconvertable Dendra2 construct would be a useful cytological tool in the study of replication timing in *N. crassa*. Therefore, I successfully introduced the hH1-Dendra2 fusion construct into *N. crassa* by transformation. The vegetative hyphae of *N. crassa* contain multiple nuclei [16]. Furthermore, this regularly branching cell type contains incomplete septa, a feature that allows the movement of organelles between hyphal compartments. Combined with the frequent fusing of hyphal filaments these characteristics enable heterokaryon formation. This means that multiple genomes can coexist inside a single mycelium at once [16]. While this fascinating feature is often capitalized upon for genomic analysis, it makes the study of the cell cycle a bit more complex in this environment. However, this photoconvertible hH1-Dendra2 fusion construct has generated an innovative method for isolating and tracking single nuclei in a

multi-nucleate environment. Additionally, the irreversible, GFP-RFP conversion offers a unique tool for tracking cellular division. Overall, I believe this construct to be multi-dimensional, and that it will prove to be useful to the study of cell cycle control and replication kinetics

Future study of the timing of origin firing in *N. crassa* could involve a sort sequencing method similar to what has been described in multiple budding yeast species [63]. Fluorescence-activated cell sorting (FACS) could be used to sort nuclei in S-phase and G2-phase from asynchronous *N. crassa* mycelia. This would remove the need for genetic manipulation before sequencing. Samples could then be subjected to deep sequencing as described [63] to measure the relative copy number of each sequence. Sequences that replicate early in S-phase would be expected to show approximately twice the copy number of those that replicate later in S-phase. From this data, a copy number profile could be generated, which would serve as a proxy for replication timing in different chromatin environments in wild-type and *dim-5* strains.

Furthermore, the rate of DNA synthesis could presumably be measured by tracking the rate of Bromodioxyuridine (BrdU) incorporation in *N. crassa* cultures with synchronized nuclear cycles [64,65]. DNA synthesis could be synchronized using a 4 hour HU block followed by a wash and transfer to Vogel's minimal media that would release stalled replication forks into S-phase at temperatures of both 25°C and 16°C as previously described in *S. cerevisiae* [61]. The purpose of releasing into S-phase at low temperature would be to slow replication rate to a point that could potentially show a more detailed profile of BrdU incorporation over time. This would be particularly valuable when comparing the kinetics of wild-type to Δ*dim-5*. Recall that previously

engineered *N. crassa* strains that have functional *Herpes simplex* thymidine kinase genes incorporated in both wild-type and Δ*dim-5* backgrounds would have to be utilized for this experiment as well [58]. The rate of DNA synthesis could then be tracked in kb/min for different regions of the genome through BrdU incorporation in synchronous cells. Samples would need to be taken at predetermined time points, and DNA could be purified through immunoprecipitation with anti-BrdU antibodies. The newly synthesized, BrdU-labeled DNA from each time point could then be sequenced as described [47,58,65].

In conclusion, development of new tools to monitor DNA replication in workable model systems is critical to our understanding of genome maintenance. **The objective of my thesis work was to develop new tools to monitor DNA replication in both wild-type and *Δdim-5* strains of *N. crassa* cells to test the hypothesis that DIM-5 is required for proper DNA replication.** I was able to successfully develop a nascent leading strand isolation and sequencing assay and a fluorescent (photoconvertible) reporter construct in *N. crassa*. However, the data return was limited for the nascent strand assay due most likely to a recently reported PCR bias. Future work must be done to get around this bias and reduce the number of PCR cycles necessary for library preparation of all samples.

There remains much to be elucidated about the role of DIM-5 in genome maintenance. It seems likely based on our recent work as well as data from other eukaryotic organisms that DIM-5 is somehow involved in the promotion of faithful DNA replication. Perhaps it does this by regulating the timing of origin firing through the control of replication factor availability or binding capacity, as do the methylatransferases

of H3K36 and H4K20. Or possibly it does so by a completely undiscovered method. Either way, there are many facets of DNA replication that require the stable, yet flexible, regulation that epigenetic modification can offer including origin selection, origin activation, and replication kinetics, and it is likely that epigenetic regulation is widespread and intricately involved in genome stability.

REFERENCES

1. Peng J. C., Karpen G. H., 2008 Epigenetic regulation of heterochromatic DNA stability. Curr. Opin. Genet. Dev. **18**: 204–211.
2. Groth A., Rocha W., Verreault A., Almouzni G., 2007 Chromatin Challenges during DNA Replication and Repair. Cell **128**: 721–733.
3. Shen H., Laird P. W., 2013 Interplay between the cancer genome and epigenome. Cell **153**: 38–55.
4. Sharma S., Kelly T. K., Jones P. A., 2009 Epigenetics in cancer. Carcinogenesis **31**: 27–36.
5. MacAlpine D. M., Almouzni G., 2013 Chromatin and DNA replication. Cold Spring Harb. Perspect. Biol. **5**.
6. Annuziato A. T., Annunziato A., 2008 DNA Packaging: Nucleosomes and Chromatin. Nat. Educ. **1**: 1.
7. Rountree M. R., Selker E. U., 2010 DNA methylation and the formation of heterochromatin in Neurospora crassa. Heredity (Edinb). **105**: 38–44.
8. Jenuwein T., Allis C. D., 2001 Translating the histone code. Science **293**: 1074–80.
9. Bannister A. J., Kouzarides T., 2011 Regulation of chromatin by histone modifications. Cell Res. **21**: 381–395.
10. Peng J. C., Karpen G. H., 2009 Heterochromatic genome stability requires regulators of histone H3 K9 methylation. PLoS Genet. **5**.
11. Lewis Z. A., Adhvaryu K. K., Honda S., Shiver A. L., Knip M., Sack R., Selker E. U., 2010 DNA methylation and normal chromosome behavior in neurospora depend on five components of a histone methyltransferase complex, DCDC. PLoS Genet. **6**.
12. Tamaru H., Selker E. U., 2001 A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. Nature **414**: 277–283.
13. Peters A. H. F. M., O'Carroll D., Scherthan H., Mechtler K., Sauer S., Schöfer C., Weipoltshammer K., Pagani M., Lachner M., Kohlmaier A., Opravil S., Doyle M., Sibilia M., Jenuwein T., 2001 Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell **107**: 323–337.
14. Galagan J. E., Calvo S. E., Borkovich K. A., Selker E. U., Read N. D., Jaffe D., FitzHugh W., Ma L.-J., Smirnov S., Purcell S., Rehman B., Elkins T., Engels R., Wang S., Nielsen C. B., Butler J., Endrizzi M., Qui D., Ianakiev P., Bell-Pedersen D., Nelson M. A., Werner-Washburne M., Selitrennikoff C. P., Kinsey J. A., Braun E. L., Zelter A., Schulte U., Kothe G. O., Jedd G., Mewes W., Staben C., Marcotte E., Greenberg D., Roy A., Foley K., Naylor J., Stange-Thomann N., Barrett R., Gnerre S., Kamal M., Kamvysselis M., Mauceli E., Bielke C., Rudd S., Frishman D., Krystofova S., Rasmussen C., Metzenberg R. L., Perkins D. D., Kroken S., Cogoni C., Macino G., Catcheside D., Li W., Pratt R. J., Osmani S. A., DeSouza C. P. C., Glass L., Orbach M. J., Berglund J. A., Voelker R., Yarden O., Plamann M., Seiler S., Dunlap J., Radford A., Aramayo R., Natvig D. O., Alex L. A., Mannhaupt G., Ebbole D. J., Freitag M., Paulsen I., Sachs M. S., Lander E. S.,

Nusbaum C., Birren B., 2003 The genome sequence of the filamentous fungus Neurospora crassa. Nature **422**: 859–868.
15. Davis R. H., Serres F. J. de, 1970 [4] Genetic and microbiological research techniques for Neurospora crassa. Methods Enzymol. **17**: 79–143.
16. Borkovich K. A., Alex L. A., Yarden O., Freitag M., Turner G. E., Read N. D., Seiler S., Bell-Pedersen D., Paietta J., Plesofsky N., Plamann M., Goodrich-Tanrikulu M., Schulte U., Mannhaupt G., Nargang F. E., Radford A., Selitrennikoff C., Galagan J. E., Dunlap J. C., Loros J. J., Catcheside D., Inoue H., Aramayo R., Polymenis M., Selker E. U., Sachs M. S., Marzluf G. A., Paulsen I., Davis R., Ebbole D. J., Zelter A., Kalkman E. R., O'Rourke R., Bowring F., Yeadon J., Ishii C., Suzuki K., Sakai W., Pratt R., 2004 Lessons from the genome sequence of Neurospora crassa: tracing the path from genomic blueprint to multicellular organism. Microbiol. Mol. Biol. Rev. **68**: 1–108.
17. Cam H. P., Sugiyama T., Chen E. S., Chen X., FitzGerald P. C., Grewal S. I. S., 2005 Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat. Genet. **37**: 809–19.
18. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J C. Y. I. H. G. S. C., 2001 Initial sequencing and analysis of the human genome. Nature **412**: 565.
19. Hoskins R. A., Carlson J. W., Kennedy C., Acevedo D., Evans-Holm M., Frise E., Wan K. H., Park S., Mendez-Lago M., Rossi F., Villasante A., Dimitri P., Karpen G. H., Celniker S. E., 2007 Sequence Finishing and Mapping of Drosophila melanogaster Heterochromatin. Science (80-.). **316**: 1625–1628.
20. Honda S., Selker E. U., 2008 Direct interaction between DNA methyltransferase DIM-2 and HP1 is required for DNA methylation in Neurospora crassa. Mol. Cell. Biol. **28**: 6044–55.
21. Tamaru H, Zhang X, McMillen D, Singh PB, Nakayama J, Grewal SI, Allis CD, Cheng X, Selker EU. 2003. Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in Neurospora crassa. Nature genetics 34:75-79.
22. Freitag M., Hickey P. C., Khlafallah T. K., Read N. D., Selker E. U., 2004 HP1 Is Essential for DNA Methylation in Neurospora. Mol. Cell **13**: 427–434.
23. Palmer J. M., Perrin R. M., Dagenais T. R. T., Keller N. P., 2008 H3K9 methylation regulates growth and development in Aspergillus fumigatus. Eukaryot. Cell **7**: 2052–2060.
24. Lundin C., North M., Erixon K., Walters K., Jenssen D., Goldman A. S. H., Helleday T., 2005 Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA double-strand breaks. Nucleic Acids Res. **33**: 3799–3811.
25. Sasaki T., Lynch K. L., Mueller C. V, Friedman S., Freitag M., Lewis Z. A., 2014 Heterochromatin Controls gammaH2A Localization in Neurospora crassa. Eukaryot Cell **13**: 990–1000.
26. Schafer K. A., 1998 The cell cycle: a review. Vet. Pathol. **35**: 461–78.
27. Rhind N., Gilbert D. M., 2013 DNA replication timing. Cold Spring Harb. Perspect. Biol. **5**.

28. Kim S. M., Dubey D. D., Huberman J. A., 2003 Early-replicating heterochromatin. Genes Dev. **17**: 330–335.
29. Hayashi M. T., Takahashi T. S., Nakagawa T., Nakayama J., Masukata H., 2009 The heterochromatin protein Swi6/HP1 activates replication origins at the pericentromeric region and silent mating-type locus. Nat. Cell Biol. **11**: 357–362.
30. Cooper G. M., Hausman R. E., 2007 *The Cell: A Molecular Approach 2nd Edition*.
31. Griffiths A., Miller J., Suzuki D., 2000 *Introduction to Genetic Analysis. 7th edition.*
32. Masai H., Matsumoto S., You Z., Yoshizawa-Sugata N., Oda M., 2010 Eukaryotic chromosome DNA replication: where, when, and how? Annu. Rev. Biochem. **79**: 89–130.
33. Martin M. M., Ryan M., Kim R. G., Zakas A. L., Fu H., Lin C. M., Reinhold W. C., Davis S. R., Bilke S., Liu H., Doroshow J. H., Reimers M. A., Valenzuela M. S., Pommier Y., Meltzer P. S., Aladjem M. I., 2011 Genome-wide depletion of replication initiation events in highly transcribed regions. Genome Res. **21**: 1822–1832.
34. Bell S. P., Dutta A., 2002 DNA replication in eukaryotic cells. Annu Rev Biochem **71**: 333–374.
35. Leonard A. C., Méchali M., 2013 DNA replication origins. Cold Spring Harb. Perspect. Biol. **5**: a010116.
36. Robinson N. P., Bell S. D., 2005 Origins of DNA replication in the three domains of life. FEBS J. **272**: 3757–3766.
37. Bell S.P., 2002 The origin recognition complex: from simple origins to complex functions. Genes Dev. **16**: 659-672
38. Bielinsky a K., Gerbi S. a, 2001 Where it all starts: eukaryotic origins of DNA replication. J. Cell Sci. **114**: 643–651.
39. Evertts A. G., Coller H. a, 2012 Back to the origin: reconsidering replication, transcription, epigenetics, and cell cycle control. Genes Cancer **3**: 678–96.
40. Liang C., Weinreich M., Stillman B., 2016 ORC and Cdc6p interact and determine the frequency of initiation of DNA replication in the genome. Cell **81**: 667–676.
41. Xouri G., Dimaki M., Bastiaens P. I. H., Lygerou Z., 2007 Cdt1 Interactions in the Licensing Process: A Model for Dynamic Spatio-temporal Control of Licensing. Cell Cycle **6**: 1549–1552.
42. Dorn E. S., Cook J. G., 2011 Nucleosomes in the neighborhood: New roles for chromatin modifications in replication origin control. Epigenetics **6**: 552–559.
43. Pryde F., Jain D., Kerr A., Curley R., Mariotti F. R., Vogelauer M., 2009 H3 K36 methylation helps determine the timing of Cdc45 association with replication origins. PLoS One **4**.
44. Reid J. L., Moqtaderi Z., Struhl K., 2004 Eaf3 regulates the global pattern of histone acetylation in Saccharomyces cerevisiae. Mol. Cell. Biol. **24**: 757–64.
45. Yokochi T., Poduch K., Ryba T., Lu J., Hiratani I., Tachibana M., Shinkai Y., Gilbert D. M., 2009 G9a selectively represses a class of late-replicating genes at the nuclear periphery. Proc. Natl. Acad. Sci. **106**: 19363–19368.

46. Fu H., Besnard E., Desprat R., Ryan M., Kahli M., Lemaitre J. M., Aladjem M. I., 2014 Mapping replication origin sequences in Eukaryotic Chromosomes. Curr. Protoc. Cell Biol. **2014**: 22.20.1–22.20.17.
47. Dewar J. M., Budzowska M., Walter J. C., 2015 The mechanism of DNA replication termination in vertebrates. Nature **525**: 345–50.
48. **McCluskey K, Wiest A, Plamann M.** The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. Journal of biosciences. 2010;35(1):119-26. PubMed PMID: 20413916.
49. Lewis ZA, Honda S, Khlafallah TK, Jeffress JK, Freitag M, Mohn F, Schubeler D, Selker EU. Relics of repeat-induced point mutation direct heterochromatin formation in Neurospora crassa. Genome Res. 2009;19(3):427-37. Epub 2008/12/19. PubMed PMID: 19092133; PubMed Central PMCID: PMC2661801.
50. Colot H. V., Park G., Turner G. E., Ringelberg C., Crew C. M., Litvinkova L., Weiss R. L., Borkovich K. A., Dunlap J. C., 2006 A high-throughput gene knockout procedure for Neurospora reveals functions for multiple transcription factors. Proc. Natl. Acad. Sci. U. S. A. **103**: 10352–10357.
51. **LangmeadB,SalzbergSL.2012.Fastgapped-readalignmentwithBowtie** 2. Nat. Methods **9:**357–359**.** http://dx.doi.org/10.1038/nmeth.1923.
52. **Thorvaldsdottir H, Robinson JT, Mesirov JP.** 2013. Integrative Genom- ics Viewer (IGV): high-performance genomics data visualization and ex- ploration. Brief. Bioinform. **14:178–192.** http://dx.doi.org/10.1093/bib /bbs017.
53. **Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP.** 2011. Integrative genomics viewer. Nat. Biotechnol. **29:24** –26. http://dx.doi.org/10.1038/nbt.1754.

54. Margolin B. S., Freitag M., Selker E. U., 1997 Improved plasmids for gene targeting at the his-3 locus of *Neurospora crassa* by electroporation. Fungal Genet. Newsl. **44**: 34–36.
55. Rodríguez-Paredes M., Esteller M., 2011 Cancer epigenetics reaches mainstream oncology. Nat. Med. **17**: 330–339.
56. Ji L., Sasaki T., Sun X., Ma P., Lewis Z. A., Schmitz R. J., 2014 Methylated DNA is over-represented in whole-genome bisulfite sequencing data. Front. Genet. **5**.
57. Sachs M. S., Selker E. U., Lin B., Roberts C. J., Luo Z., Vaught-Alexander D., Margolin B. S., 1997 Expression of herpes virus thymidine kinase in Neurospora crassa. Nucleic Acids Res. **25**: 2389–2395.
58. Feng W., Collingwood D., Boeck M. E., Fox L. A., Alvino G. M., Fangman W. L., Raghuraman M. K., Brewer B. J., 2006 Genomic mapping of single-stranded DNA in hydroxyurea-challenged yeasts identifies origins of replication . Nat. Cell Biol. **8**: 148–155.
59. Sabatinos SA. 2010. Replication Fork Stalling and the Fork Protection Complex. Nature Education 3.
60. Katou Y., Kanoh Y., Bando M., Noguchi H., Tanaka H., Ashikari T., Sugimoto K., Shirahige K., 2003 S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature **424**: 1078–1083.
61. Leach T. J., Chotkowski H. L., Wotring M. G., Dilwith R. L., Glaser R. L., 2000 Replication of heterochromatin and structure of polytene chromosomes. Mol. Cell. Biol. **20**: 6308–16.

62. Müller C. A., Nieduszynski C. A., 2012 Conservation of replication timing reveals global and local regulation of replication origin activity. Genome Res. **22**: 1953–1962.
63. Cha R. S., Kleckner N., Abraham R., Zhou B. B., Elledge S. J., Zhao X., Chabes A., Domkin V., Thelander L., Rothstein R., Desany B., Alcasabas A. A., Bachant J. B., Elledge S. J., Xu Y., Baltimore D., Baker B., Carpenter A., Grushcow J. M., Holzen T. M., Park K. J., Weintert T., Licheten M., Bishop D. K., Shirahige K., Hennessy K., Lee A., Chen E., Botstein D., Schlegel R., Pardee A., Brown E., Baltimore D., Blat Y., Kleckner N., Borde V., Wu T.-C., Lichten M., Raghuraman M., Waizenegger I., Hauf S., Meinke A., Peters J., Thrower D., Bloom K., Lopes M., Tercero J., Diffley J., Laird C., Hansen R., Canfield T., Lamb M., Gartlers S., Shibahara K.-I., Stillman B., Toth A., Ciosk R., Uhlmann F., Galova M., Schleiffer A., Nasmyth K., Skibbens R. V., Corson L. B., Koshland D., Hieter P., Le S., Davis C., Konopka J., Sternglanz R., Mathews C., Reddy G., Pardee A., Cha R., Weiner B., Keeney S., Dekker J., Kleckner N., Yang D., Kastan M., Poloumienko A., Dershowitz A., De J., Newlon C., 2002 ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science **297**: 602–6.
64. Lengronne A., Pasero P., Bensimon A., Schwob E., 2001 Monitoring S phase progression globally and locally using BrdU incorporation in TK(+) yeast strains. Nucleic Acids Res. **29**: 1433–42